

Topical tacrolimus in atopic dermatitis: Effects of long-term treatment on skin and respiratory symptoms

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Academic dissertation

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The Cover

The background silhouette shows the fungus-like bacterium *Streptomyces tsukubaensis* from which tacrolimus was first isolated. Modified from the original photograph by Sakari Reitamo. Reprinted with the owner's permission.

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To Juha and Veera

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are cited in the text by their Roman numerals:

- I. Remitz A, Kyllönen H, Granlund H, Reitamo S: Tacrolimus ointment reduces staphylococcal colonization of atopic dermatitis lesions. *J Allergy Clin Immunol* 107:196-197, 2001.
- II. Kyllönen H, Remitz A, Mandelin JM, Elg P, Reitamo S. Effect of one-year intermittent treatment with topical tacrolimus monotherapy on skin collagen synthesis in patients with atopic dermatitis. *Br J Dermatol* 150:1174-1181, 2004.
- III. Kyllönen H, Malmberg P, Remitz A, Ryttilä P, Metso T, Helenius I, Haahtela T, Reitamo S. Respiratory symptoms, bronchial hyper-responsiveness, and eosinophilic airway inflammation in patients with moderate-to-severe atopic dermatitis. *Clin Exp Allergy* 36:192-197, 2006.
- IV. Virtanen H, Kari O, Kari M, Reitamo S. Tacrolimus ointment in the treatment of atopic blepharoconjunctivitis. A retrospective study. *Acta Ophth Scand.* 84:693-695, 2006.
- V. Virtanen H, Remitz A, Malmberg P, Ryttilä P, Metso T, Haahtela T, Reitamo S. Topical tacrolimus for atopic dermatitis. Does it benefit the airways? A four-year follow-up. *J Allergy Clin Immunol* 120:1464-1466, 2007.

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Some previously unpublished data are also presented.

ABBREVIATIONS

AD	Atopic dermatitis
BHR	Bronchial hyper-responsiveness
BSA	Body surface area
CLA	Cutaneous lymphocyte-associated antigen
FcεRI/ FcεRII	High-affinity receptor for IgE type I/II
FK506	Tacrolimus molecule
HSV	<i>Herpes Simplex</i> virus
IDEC	Inflammatory dendritic epidermal cell
IFN	Interferon
IgE, S-IgE	Immunoglobulin E, Serum immunoglobulin E
IL	Interleukin
LC	Langerhans cell
MMP	Matrix metalloproteinase
NF-AT	Nuclear factor for activated T cells
PEF	Peak expiratory flow
PICP	Carboxyterminal propeptide of type I collagen
PINP / PIIINP	Aminoterminal propeptide of type I / III collagen
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SBF	Suction blister fluid
SPT	Skin prick tests
TEWL	Transepidermal water loss
TIMP	Tissue inhibitor of metalloproteinase
TGF-β	Transforming growth factor β
Th1 / Th2	T helper cells type 1 / type 2
UV	Ultraviolet
VAS	Visual analogue scale

ABSTRACT

Objective: Patients with atopic dermatitis often have a poor long-term response to conventional topical or systemic treatments. They suffer from staphylococcal superinfections, skin atrophy due to corticosteroid use, and may have asthma and allergic rhinitis. The association between atopic skin and airways has been thought to result from epicutaneous sensitization through the inflamed skin, which leads to systemic T cell responses and airway hyperreactivity. Only a few, usually short-term, studies have addressed the effects of different treatments of atopic dermatitis on these problems. Tacrolimus ointment is the first topical compound suitable for long-term treatment. The aim of this thesis was to evaluate the effects of long-term topical tacrolimus treatment on cutaneous staphylococcal colonization, collagen synthesis, and symptoms and signs of asthma and allergic rhinitis.

Methods: Patients with moderate-to-severe atopic dermatitis were treated with intermittent 0.1% tacrolimus ointment in prospective, open, long-term studies lasting for 6 to 48 months. In Study I, staphylococcal colonization of their skin was followed by bacterial cultures for 6 to 12 months. In Study II, skin thickness and collagen synthesis were followed by skin ultrasound and procollagen I and III propeptide concentrations of the suction blister fluid samples for 12 to 24 months and compared with a group of atopic dermatitis patients treated with topical corticosteroids and with a group of healthy subjects. Study III was a cross-sectional study including atopic dermatitis patients and healthy controls. The occurrence of respiratory symptoms was determined with a questionnaire, bronchial hyper-responsiveness by bronchial histamine challenge, and sputum eosinophilia by induced sputum test. Atopy status was assessed by skin prick test reactivity and serum immunoglobulin E concentration. In Study V, the same parameters as in Study III were assessed in atopic dermatitis patients before and after 12 to 48 months of topical tacrolimus treatment. Study IV was a retrospective follow-up of the effect of tacrolimus 0.03% ointment on severe atopic blepharoconjunctivitis and conjunctival cytology samples.

Results: The clinical response of atopic dermatitis to topical tacrolimus was very good in all studies ($p \leq 0.008$). Staphylococcal colonization decreased significantly, and the effect was sustained throughout the study period ($p = 0.01$). Skin thickness ($p < 0.001$) and markers of collagen synthesis ($p < 0.001$) increased in the tacrolimus-treated patients significantly, whereas they decreased or remained unchanged in the corticosteroid-treated controls. Symptoms of asthma and allergic rhinitis ($p < 0.0001$), bronchial hyper-responsiveness ($p < 0.0001$), and sputum eosinophilia ($p < 0.0001$) were significantly more common in patients with atopic dermatitis than in healthy controls, especially in subjects with positive skin prick tests or elevated serum immunoglobulin E. During topical tacrolimus treatment the asthma and rhinitis ($p = 0.005$ and $p = 0.002$) symptoms

and bronchial hyper-responsiveness ($p=0.02$) decreased significantly in patients with atopic dermatitis, and serum immunoglobulin E and sputum eosinophils showed a decreasing trend in atopic dermatitis patients with a continuously good treatment response. Treatment of eyelid dermatitis with tacrolimus ointment resulted in a marked clinical response and a significant decrease in eosinophils, lymphocytes, and neutrophils in the conjunctival cytology samples. No significant adverse effects or increase in skin infections occurred in any study.

Conclusions: The studies included in this thesis, except the study showing an increase in skin collagen synthesis in tacrolimus-treated patients, were uncontrolled, warranting certain reservations. The results suggest, however, that tacrolimus ointment has several beneficial effects in the long-term intermittent treatment of atopic dermatitis. Tacrolimus ointment efficiently suppresses the T cell-induced inflammation of atopic dermatitis. It has a normalizing effect on the function of the skin measured by the decrease in staphylococcal colonization. It does not cause skin atrophy as do corticosteroids but restores the skin collagen synthesis in patients who have used corticosteroids. Tacrolimus ointment has no marked systemic effect, as the absorption of the drug is minimal and decreases along with skin improvement. The effects on the airways—decrease in bronchial hyper-responsiveness and respiratory symptoms—can be speculated to be caused by the decrease in T cell trafficking from the skin to the respiratory tissues as the skin inflammation resolves, as well as inhibition of epicutaneous invasion of various antigens causing systemic sensitization when the skin barrier is disrupted as in atopic dermatitis. Patients with moderate-to-severe atopic dermatitis seem to benefit from efficient long-term treatment with topical tacrolimus.

INTRODUCTION

Atopic dermatitis (AD) is a common disease of unknown etiology. The name atopic eczema/dermatitis syndrome (AEDS) was suggested in a revised nomenclature in 2001 and is used by some authors, especially allergists (Johansson et al. 2001), but this thesis will use the term AD. In AD clinical manifestations, both genetic and environmental factors play a role. AD is characterized by itchy, dry skin, a chronically relapsing course, and susceptibility to cutaneous infections. Approximately 80% of patients with AD show an IgE-mediated allergy to exogenous allergens such as pollen, animal dander, house dust mite, or food allergens. A patient with AD often suffers from asthma or allergic rhinitis or both.

The therapeutic options for AD can be divided in several ways: topical vs. systemic, first-line vs. second-line, and treatments suitable for monotherapy vs. combination therapy. Topical corticosteroids have been the mainstay of first-line treatment of atopic dermatitis and can be used as monotherapy. Systemic corticosteroids, natural or artificial ultraviolet (UV) therapies, and immunosuppressive agents such as azathioprine, methotrexate, and ciclosporin are second-line therapies used in combination with topical corticosteroids. Major problems with topical corticosteroids include tachyphylaxis and skin atrophy due to suppression of collagen synthesis of the connective tissue. For this reason, topical corticosteroids are indicated for short-term treatment, which is contradictory to the fact that AD is a chronic disease. Periodic short-term use of topical corticosteroids often leads to insufficient long-term disease control. The second-line treatments of AD diminish the need for topical corticosteroids but do not replace them. Although AD is a very common and chronic disease, efficacy in placebo-controlled studies has been shown only for topical corticosteroids, oral ciclosporin, and azathioprine. Furthermore, only few long-term studies involve with topical corticosteroids.

The topical calcineurin inhibitors—tacrolimus ointment and pimecrolimus cream—represent a new non-corticosteroid-based treatment possibility for AD. In several long-term clinical settings they have proven safe and efficient. In these studies, tacrolimus ointment has mainly served as monotherapy, whereas pimecrolimus cream has served as baseline therapy with corticosteroids allowed for disease exacerbations. They thus represent the first potential candidates to replace topical corticosteroids as the primary treatment for AD.

This thesis elucidates the background of AD and its treatments in general, and concentrates on the effects of long-term topical tacrolimus monotherapy on major problem areas in the treatment of AD, namely *Staphylococcus aureus* colonization, collagen synthesis of the skin, and treatment of thin and sensitive skin areas such as the eyelids; and finally, it examines the interaction between AD and the other atopic diseases asthma and allergic rhinitis.

REVIEW OF THE LITERATURE

Atopic dermatitis

Clinical features

Atopic dermatitis (AD), or atopic eczema, is a common, chronic, inflammatory skin disease. AD prevalence is dependent on the criteria used. Its incidence has been increasing during the last decades in Western countries (Taylor et al. 1984, Grize et al. 2006, Romano-Zelekha et al. 2007). The prevalence of AD is 10 to 20% in children and 1 to 3% in adults in temperate developed countries (Schultz-Larsen et al. 1996). In children, 60% of AD is diagnosed under the age of one year (Kay et al. 1994). Typical features are dry skin, erythema, scaling, lichenification, and itching which causes excoriations through scratching (Hanifin & Rajka 1980). AD has a chronic or chronically relapsing course with remissions and exacerbations of variable length (Hanifin & Rajka 1980). The lesions have a typical age-dependent distribution pattern. In infants, nummular or seborrhoeic type eczema occurs on the cheeks, chin, and trunk. In 1 to 4 year-old children, affected areas include extensor and sometimes flexural sides of the extremities, the hands, face, neck, and perioral area. In 4 to 16-year-old children, flexural eczema predominates, with face, hands, feet, and gluteal area affected. In adults, eczema most often occurs in the face, neck, upper body, and extremities (Bohme et al. 2001). Some patients develop an erythrodermic AD affecting almost the whole body surface area (Thestrup-Pedersen 2000). Roughly 70 to 80% of the patients show IgE-mediated hypersensitivity to common aeroallergens or foods (Wütrich 1978). The patients often develop also other atopic diseases: asthma and allergic rhinoconjunctivitis (Spergel & Paller 2003).

Diagnosis and differential diagnosis

AD is a clinical diagnosis based on several features. Hanifin and Rajka (1980) were the first to develop a diagnosis of AD based on defined criteria (Hanifin & Rajka 1980). These criteria have been modified and simplified for use in epidemiological studies (Schultz-Larsen et al. 1996, Williams et al. 1994, Diepgen et al. 1996, Bos et al. 1998). A recent study found that in a clinical setting the sensitivity and specificity, as well as positive and negative predictive values of the criteria proposed by Hanifin and Rajka were superior to the UK Working Party's criteria by Williams et al. (1994) (De et al. 2006). According to Hanifin and Rajka, three of the four main criteria—pruritus, typical eczema lesions in typical skin areas, chronic or chronically relapsing course, and patient's personal or family history of AD or other atopic diseases, especially in

childhood—must be met for the diagnosis of AD (Hanifin & Rajka 1980). In addition, several minor criteria are non-specific for AD, of which at least three are required: dry skin, elevated serum immunoglobulin E (s-IgE) or positive skin prick test results for aeroallergens or food allergens, susceptibility to skin infections, susceptibility to unspecific hand or foot eczema, Dennie-Morgan lines under the lower lids, white dermographism, or keratosis pilaris (Hanifin & Rajka 1980). Criteria also exist for estimation of disease severity. The Rajka & Langeland criteria grade AD to mild, moderate, and severe disease by summing the scores for extent, course, and intensity (by itch and loss of sleep) of AD (Rajka & Langeland 1989).

The typical AD lesions are dry, scaling, erythematous papules and plaques, which in chronic lesions become lichenified. The histopathologic findings in AD include changes typical for any type of eczema: spongiosis, epidermal hyperplasia, thickening of the papillary dermis, parakeratosis, and a superficial perivascular inflammatory infiltrate consisting mainly of T lymphocytes (Akdis et al. 2006).

The differential diagnosis of atopic dermatitis includes seborrheic eczema, psoriasis, nummular and infectious dermatitis, contact dermatitis, asteatotic eczema, dermatitis herpetiformis, dyshidrosis, and (especially in children) food allergy, scabies, ichthyosis vulgaris, and Netherton's syndrome. Netherton's syndrome is an autosomal recessive skin disorder characterized by ichthyosis and atopy, and highly increased skin permeability (Akdis et al. 2006).

Etiology and pathogenesis of atopic dermatitis

Genetics

The etiology of AD is unknown, but several features of its pathogenesis are known. The genetic predisposition for AD is strong. Parental, especially maternal (Morar et al. 2006), AD has been a greater risk factor for children's AD than is either parental asthma or allergic rhinitis (Dold et al. 1992). In twin studies, the concordance rate for AD has been two- to three-fold in monozygous twins compared with the rate of dizygous twins (Hopp et al. 1984, Schultz-Larsen 1993). Its inheritance is complex and includes multiple gene interactions and environmental influences. AD, asthma, and elevated serum IgE have been linked to almost every chromosome, but no single gene can be implicated as the gene for the development of AD (Cork et al. 2006).

In candidate gene studies, significant associations with AD have appeared in genes encoding for example high-affinity IgE receptor (FcεRI), mast cell chymase, transforming growth factor β (TGF-β), toll-like receptors 2 and 9, interleukins 4, 13, and 18, granulocyte-macrophage colony-stimulating factor, as well as the chemokines RANTES and eotaxin (Morar et al. 2006). A polymorphism variant of the gene implicated in Netherton disease, SPINK5, encoding a serine protease inhibitor expressed in the outer layers of the skin, has been associated with AD. This gene

probably plays a protective role against allergens that are serine proteases (Kato et al. 2003). Also, some gene polymorphisms implicated in innate immunity, for example, a polymorphism resulting in functional impairment of the intracellular receptor for lipopolysaccharide involved in activation of a nuclear factor necessary for T cell activation has been associated with risk for AD (Kabesch et al. 2003).

A genetic association with AD has appeared in mutations of the gene cluster encoding the epidermal differentiation complex in chromosome 1q21 (Segre 2006). The strongest evidence of genetic barrier dysfunction predisposing to AD has been found in the FLG gene encoding the filaggrin protein—the same gene in which mutations are linked in ichthyosis vulgaris. Null mutations leading to filaggrin deficiency occur in about 6 to 10% of the European-origin population as a semidominant trait and with incomplete penetrance (Smith et al. 2006, Weidinger et al. 2007). In studies looking at AD and filaggrin mutations, 44% of subjects with one mutant allele and 76% with both filaggrin alleles mutant had a diagnosis of AD (Palmer et al. 2006). FLG mutations have been present in 25% of patients with IgE-associated but only 12% of patients with non-IgE-associated AD. They have also been associated with asthma in the patients with AD but not with asthma without AD (Marenholz et al. 2006, Weidinger et al. 2007). Furthermore, these mutations associate with AD that starts early, is more severe, and persists into adulthood (Barker et al. 2007, Stemmler et al. 2007, Weidinger et al. 2006).

Skin barrier function

One of the most crucial functions of the skin is to form a barrier against the microbes, irritants, and allergens of the exterior world. This barrier function is impaired in AD. Peptides with a molecular mass over 500 Da do not penetrate healthy normal skin, whereas in AD, environmental allergens of up to 20 kDa may penetrate the skin (Bos and Meinardi 2000). This makes the skin susceptible to environmental factors such as detergents, irritants, allergens, microbial toxins, and physical or psychological stress.

The epidermis is formed of layers of keratinocytes which divide in the basal layer next to the basement membrane, increasing their keratine content and flattening on their way to the surface of the skin (Segre 2006). The skin barrier is formed in the outermost part of the epidermis called the stratum corneum by compact flat layers of keratinocytes called corneocytes and their surrounding hydrophobic lipid matrix (Segre 2006). Active functional filaggrin is needed to condense the corneocytes (Palmer et al. 2006), whereas the water-retaining properties of the hydrophobic lipid matrix are provided by structural proteins such as the ceramides (Segre 2006).

Defects of epidermal differentiation such as filaggrin mutations weaken the epidermal barrier and predispose patients with AD to epicutaneous allergic sensitization as well as to physical, microbial, and irritant skin damage. This barrier-damage activates keratinocytes to produce inflammatory cytokines and starts the inflammatory cycle leading to T cell activation, IgE-production, and clinical AD (Vickery 2007, Segre 2006).

The skin of an atopic person is deficient in ceramides even in the non-lesional areas, and this leads to increased transepidermal water loss (TEWL) and xerosis (dry skin) (Leung et al. 2004). TEWL can serve as a marker of skin barrier function and be measured with a special device (Pinnagoda et al. 1990). The reduced amount of ceramides in the skin elevates susceptibility to irritants and leads to increased antigen absorption through the skin (Segre 2006). In one study, ceramide-containing creams resulted in improved barrier function in healthy tape-stripped skin and also produced improvement in TEWL and eczematous lesions in AD patients when added to their standard therapy (Chamlin et al. 2002).

Additionally, the skin barrier can be disrupted or weakened by inflammation. In the inflamed AD skin, keratinocyte apoptosis increases through direct T cell cytotoxicity and indirectly through soluble T cell products such as interferon gamma (IFN- γ), which causes spongiosis of the epidermis and thus disrupts the skin barrier (Trautmann et al. 2000). Low expression of TGF- β and high expression of nitric oxide synthase of the keratinocytes have also been linked to the pathogenesis of AD (Novak et al. 2003).

Another important factor weakening the barrier function of AD skin is a deficiency in antimicrobial peptides such as β -defensins and cathelicidin that protect the skin against bacteria, viruses, and fungi (Ong et al. 2002, Nomura et al. 2003). This makes the patients susceptible to skin infections with e.g. *Staphylococcus aureus*, *Malassezia furfur* (formerly known as *Pityrosporum ovale*), and *Herpes simplex virus*.

Pathophysiology of AD

The complicated pathogenetic pathway leading to AD begins when antigens such as aeroallergens, food antigens, autoantigens, or bacterial superantigens enter the skin. They are then taken up by antigen-presenting cells such as Langerhans cells (LC) and inflammatory dendritic epidermal cells (IDEC) both carrying the high-affinity IgE receptor Fc ϵ RI and IgE molecules (Fc ϵ RI+/ IgE+ cells) (Novak et al. 2003). The Fc ϵ RI-bound IgE facilitates the capture and internalization of allergens, which are then processed and presented to T cells. This results in an increase in intracellular Ca²⁺ which activates calcineurin, a phosphatase enzyme, within the T cells. Calcineurin then dephosphorylates the nuclear factor of activated T cells (NF-AT). After that, the dephosphorylated NF-AT is able to move to the nucleus of the thus activated T cell and induce gene transcription of various inflammatory cytokines such as interleukins (IL-2, IL-4, IL-5, IL-13), tumor necrosis factor α , and IFN- γ . The cytokines in turn activate more T cells and cause IgE production in B cells, leading to a vicious circle (Novak et al. 2003, Homey et al. 2006, Leung et al. 2004, Boguniewicz & Leung 2006).

The T cells found in the lesional and to a lesser extent non-lesional skin are positive for cutaneous lymphocyte-associated antigen (CLA) which causes selective migration of T cells to the skin. Subjects with AD have increased amounts of these skin-homing T cells. CLA is mainly expressed in T helper type 1 (Th1) cells during the normal differentiation process of T cells, whereas in T helper type 2 (Th2) cells it can

be induced by IL-2 and bacterial superantigen stimulation. The number of T cells is increased because of inhibition of their apoptosis by cytokines and extracellular matrix components (Homey et al. 2006).

The AD pathway is orchestrated by cytokines and chemokines secreted by the LCs, IDECs, T cells, keratinocytes, eosinophils, and mast cells. In acute AD, Th2 cells producing predominantly IL-4 characterize the inflammatory infiltrate. Th2 cells are also found in immediate hypersensitivity reactions. After 24 to 48 hours, the T cell infiltrate switches to IFN- γ -producing Th1 cells, which take part in immune reactions to infectious agents and in chronic inflammatory disorders such as psoriasis. This switch is believed to be initiated by IL-12 and IL-18 secreted by eosinophils and IDECs. Macrophages make up a significant part of the cellular infiltrate in chronic AD. Key cytokines other than the IL-4 and IFN- γ secreted by activated T cells are the IL-5 and IL-13 seen in both acute and chronic phases of AD. Tissue and blood eosinophilia is also typical for atopic diseases and AD. IL-5 is important for eosinophilia, as it prevents eosinophil apoptosis in both IgE-associated and non-IgE-associated forms of AD, whereas IL-13 activates B cells and their IgE production in the IgE-associated AD (Novak et al. 2003, Homey et al. 2006, Leung et al. 2004, Boguniewicz & Leung 2006).

Fc ϵ RI+ IgE+ LCs are crucial for AD, as their presence is required to provoke eczematous skin lesions in aeroallergen-induced patch testing (Laouini et al. 2003). These cells may also migrate to the lymph nodes and stimulate naive T cells to develop into Th2 cells and increase the migratory capacity of IDECs. Fc ϵ RI-activated IDECs secrete proinflammatory cytokines and chemokines that probably amplify the atopic inflammatory reaction. They also prime naive T cells into IFN- γ secreting Th1 cells, and they release IL-12 and IL-18, which together might lead to the switch of the T cell profile from Th2 to Th1 (Novak et al. 2003).

A central feature in AD is pruritus but histamine and other molecules behind the immediate hypersensitivity reactions have not been able to explain it (Ständer et al. 2003). Its etiology in AD seems to be more complicated, and several molecules such as neuropeptides, proteases, cannabinoids, opioids, kinins, cytokines, biogenic amines, and neurotransmitters probably play a role (Ständer et al. 2003). IL-31, a recently described cytokine expressed by Th2 cells, has induced pruritus and dermatitis in mice (Sonkoly et al. 2006), offering one explanation for the etiology of itch in AD.

Microbes and atopic dermatitis

As the skin barrier function in AD is weakened, and the skin is deficient in antimicrobial peptides such as β -defensins and cathelidins (Ong et al. 2002), various microbes are able to colonize the skin and cause secondary infections. The clinically relevant microbes in AD are *Staphylococcus aureus*, *Herpes simplex virus* (HSV), and *Malassezia* (formerly known as *Pityrosporum*) yeast species.

Skin colonization with *Staphylococcus aureus* bacteria is found in about 90% of the AD patients compared with approximately 5% of healthy controls (Aly 1980). It is the most common cause of secondary infections of the AD skin. *S. aureus* shows

increased adhesion to the eczematous skin via fibronectin and fibrinogen (Cho et al. 2001). The bacteria also produce superantigens (S. enterotoxin A, S. enterotoxin B, toxic shock syndrome toxin-1) which are important effectors in AD (Bunikowski et al. 2003). They cause *S. aureus*-specific IgE production that correlates with disease severity (Leung et al. 1993). Superantigens also cause nonspecific IgE production, activate T cells, B cells, and macrophages, and stimulate their proliferation (Ou et al. 2004). Lately, they have been found to induce chemokines such as CCL1 and CCL18, which bind to CLA-positive T cells in peripheral blood, thus possibly playing a role in T cell homing to the skin (Pivarcsi et al. 2004, Gombert et al. 2005). The superantigens seem to reduce the immunosuppressive activity of certain immunosuppressive regulatory T cells, which may, in turn, increase the inflammatory T cell activation (Ou et al. 2004). They are also known to induce corticosteroid resistance complicating the treatment of atopic diseases (Gould et al. 2007).

HSV infections can lead to the acute disseminated viral infection *eczema herpeticum*, which often requires hospitalization (Novak & Bieber 2005). Smaller, local lesions with or without typical blisters are another typical form of the infection. HSV superinfection is a risk factor for acute and severe exacerbations of AD (Bork & Brauninger 1986). In AD patients, immunosuppressive treatment (corticosteroids, calcineurin inhibitors) is a potential trigger of HSV infections (Novak & Bieber 2005). β -defensins have shown some antiviral activity against HSV (Ong et al. 2002). Other mechanisms favoring HSV overgrowth in AD include predisposition to Th2 type responses and impaired recruitment of plasmacytoid dendritic cells to the skin, which leads to reduced amounts of antiviral type 1 IFN- α and IFN- β (Wollenberg et al. 2003, Novak & Bieber 2005).

Malassezia yeast species colonize the skin of 90% of AD patients compared with 35% of healthy controls, especially the sebaceous areas face, scalp, and upper body. Species associated with AD include *Malassezia globosa*, *sympodialis*, *restricta*, and *furfur*. (Scheynius et al. 2003) Their role in AD exacerbations has been controversial despite the fact that specific IgE antibodies to *Malassezia* species can be found in AD patients but not in healthy controls (Roll et al. 2004, Baker 2006).

Allergic vs. nonallergic atopic dermatitis

About 70 to 80% of patients with AD are considered to have classical, i.e. IgE-associated or allergic, AD because they show elevated s-IgE levels or positive skin prick test results for aeroallergens or food allergens, whereas the remaining 20 to 30% never show this kind of IgE-mediated sensitization and are considered to have non-IgE-associated or nonallergic AD (Wütrich 1978). IgE-mediated sensitization may not yet be evident in infants or young children but it develops with increasing age. After a thorough or repeated allergologic work-up, some nonallergic AD patients may be reclassified as having allergic AD (Novak & Bieber 2003). It is unclear whether the allergic and nonallergic atopic diseases are truly separate diseases or represent different degrees of severity of the same disease. The nonallergic type is usually clinically milder

and has a lower risk for asthma and allergic rhinitis and is characterized by less eosinophilia and fewer CLA-positive T cells as well as FcεRI-positive cells (Wütrich et al. 2002, Novak & Bieber 2003). Recent findings suggest that the nonallergic AD is associated with sensitization to microbial antigens such as *S. aureus*, *Malassezia* species (*Pityrosporum*), and *Candida albicans* (Novak et al. 2003), as association also seen in the allergic type. Tests to detect IgE-mediated sensitization to these microbial antigens are not included in the routine tests for allergic AD.

Prognosis and risk factors

Although the genetic component in AD is strong, it does not explain the strong increase in the prevalence of AD in a few decades suggesting that various environmental factors contribute to the development of this disease (Novak et al. 2003). A recent review suggested that susceptibility to AD depends on the number of primary genetic defects and of environmental stress factors, so that minor or only a few genetic defects would require several environmental factors to launch AD, whereas severe or multiple genetic defects of the skin barrier would require lighter environmental load to initiate AD (Cork et al. 2006).

General risk factors for AD and atopic diseases include positive family history, especially maternal atopy, maternal smoking, low birth weight, early infection with respiratory syncytial virus (a risk factor for asthma), and early sensitization to aeroallergens (Arruda et al. 2005, Novak et al. 2003, Akdis et al. 2006). Possible protective factors against AD include early exposure to certain infections or infective agents, such as endotoxin from the soil or animal contacts (Elston 2006). The fetal lymphocytes are skewed toward a Th2 profile, and microbial stimulation of the immune system would switch it toward a Th1 profile in nonatopic subjects, whereas decreased bacterial stimulation would lead to a predominantly Th2 type lymphocyte profile (Elston 2006, Novak et al. 2003). At present, the data of this hygiene theory is controversial (Zutavern et al. 2005). Addition of probiotics to the diet has also shown some protective effect (Boyle & Tang 2006), whereas the benefit of breast feeding still seems questionable (Chan-Yeung & Becker 2006).

The natural history of AD from childhood to adulthood is not very well understood; the definition and length of remissions vary, and the impact of treatment—particularly topical corticosteroids—on the course of the disease is unknown (Williams 2000). Typically, the childhood eczema clears in about half the children before school age but has a tendency to relapse later in the adolescence or adulthood possibly after a remission of several years (Williams & Strachan 1998, Rystedt 1985, Lammintausta et al. 1991). For this reason, the natural history of AD is not easy to evaluate. Early sensitization to aeroallergens and severe eczema in childhood are risk factors for AD's persisting from childhood into later ages (Ben-Gashir et al. 2004). Filaggrin gene mutations also predispose to persistent AD (Barker et al. 2007).

Atopy-related diseases in atopic dermatitis patients

Asthma and allergic rhinitis

Atopic respiratory diseases include asthma and allergic rhinitis. Asthma is determined as a common chronic inflammatory disease of the lower airways (Global initiative for asthma, GINA; Global Strategy for Asthma Management and Prevention 2006). Like AD, it can be divided, according to its IgE-mediated sensitization to common aeroallergens, into an allergic and a non-allergic form (Johansson 2004). Allergic asthma is a common disease affecting children in particular, whereas the non-allergic type can begin at any age, usually in adulthood (Reed 2006). Symptoms of asthma include wheeze, dyspnoea especially induced by physical stress or exposure to aeroallergens or small air-borne particles, and prolonged cough (Bousquet et al. 2000). Allergic rhinitis is characterized by one or more symptoms including sneezing, itching, nasal congestion, and rhinorrhea. It can be divided into seasonal allergic rhinitis associated with pollen exposure and perennial allergic rhinitis that lasts for at least 9 months of the year. Mixed types are also seen (Skoner 2001, Bousquet et al. 2001).

Asthma is characterized by reversible airway obstruction, increased bronchial hyper-responsiveness to external stimuli, eosinophilic inflammation of the lower airways, and airway wall remodeling (Bousquet et al. 2000, GINA). Reversible obstruction of the lower airways can be demonstrated by spirometry with a bronchodilator test and by follow-up of peak expiratory force (PEF) values, by which diurnal variation in results and the effect of a bronchodilating agent are evaluated. BHR can be measured with a bronchial challenge test with bronchoconstricting stimuli like histamine or methacoline. Eosinophilic inflammation and remodeling (thickening of the airway wall and hypertrophy of the smooth muscle layer and of mucous glands) of the lower airways is apparent in mucosal biopsies of the bronchi. In clinical settings, airway inflammation is estimated by use of noninvasive methods like an induced sputum test or measurement of increased exhaled air like nitric oxide (NO) (Bousquet et al. 2000, Frieri 2005).

Asthma and allergic rhinitis share common epidemiologic and pathophysiologic features. The prevalence of allergic rhinitis is at least three-fold of the prevalence of asthma (Togias 2003, Braunstahl & Hellings 2006). In Finnish adolescents and young adults, the prevalence of allergic rhinitis is approximately 26%, and the prevalence of asthma is approximately 5% (Huurre et al. 2004). Of adult patients with asthma or allergic rhinitis, 80% actually have both diseases and almost all adult patients with asthma also have allergic rhinitis (Spergel 2005, Togias 2003). In patients with asthma, a strong correlation exists for clinical disease activity between asthma and allergic rhinitis, so that patients with severe asthma usually have more severe allergic rhinitis do those with rhinitis alone (Togias 2003). Similar interactions between rhinitis and asthma occur also in nonallergic subjects (Togias 2003).

Both asthma and allergic rhinitis involve mucosal infiltrates containing eosinophils, mast cells, and Th2 type lymphocytes with corresponding cytokine and chemokine expression and production (Togias 2003, Jeffery & Haahtela 2006). Patients with allergic rhinitis often present with BHR and eosinophilia of the lower airway mucosa (Spergel 2005). Cellular infiltrates, airway hyper-responsiveness, and early and late phase reactions similar to those in nasal allergic reactions occurred in the bronchial mucosa in nasal allergen provocation tests (Togias 2003). Patients with allergic rhinitis alone also show a thickened lamina reticularis and mucosal eosinophilia of the lower airways, features typical of asthma (Togias 2003, Jeffery & Haahtela 2006). For these reasons, asthma and allergic rhinitis are nowadays regarded as one syndrome, “combined allergic rhinitis asthma syndrome”, involving different target organs and having different degrees of severity (Taramarcaz & Gibson 2004). Allergic rhinitis alone is considered as the commonest and mildest form of the syndrome, and the patients with asthma can be viewed as a subpopulation of patients with allergic rhinitis so that allergic rhinitis is accompanied by BHR and clinical asthma by increasing rhinitis severity (Togias 2003, Braunstahl & Hellings 2006).

Several mechanisms by which allergic rhinitis influences or causes asthma have been suggested: When the nasal mucosa is inflamed, the protective functions of the nose regarding inhaled air (humidification, warming, and removal of small particles and irritant gases) are bypassed and the bronchial mucosa is directly exposed to these factors (Togias 2003). On the other hand, inflammation in the nasal mucosa is thought to be systemically propagated to the lower airways. This immunologic propagation seems to play a dual role: It feeds back to the original nasal inflammation explaining the strong correlation of clinical disease activity between asthma and allergic rhinitis (Togias 2003, Braunstahl & Hellings 2006).

The natural history of asthma is variable, depending on several factors. About half the patients with childhood asthma will be in clinical remission by their teens, but they may still exhibit bronchial hyper-responsiveness and airway eosinophilia, which may predispose them to asthma relapse later in life (van Den Toorn et al. 2000). The risk for persistent asthma is increased by severe, recurrent wheeze and several respiratory infections before school age, by early sensitization to aeroallergens, and by a positive family history for asthma (Castro-Rodriguez et al. 2000). When allergic asthma starts at school age, it is more likely to persist but is not usually very severe and not likely to progress, whereas nonallergic asthma is usually more severe in nature and very persistent (Reed 2006).

Anti-inflammatory treatment with inhaled glucocorticosteroids is the mainstay of treatment of persistent asthma. Short- and long-acting bronchodilating agents are help to relieve symptoms. Theophylline or systemic agents may be added for severe disease. When the anti-inflammatory treatment is started as early and efficiently as possible, disease progression can be halted and this may often be followed by intermittent treatment for exacerbations for instance during pollen season or respiratory infection (Jeffery & Haahtela 2006, GINA). Symptoms of allergic rhinitis are managed with

antihistamines and nasal decongestants, and inflammation is controlled by intranasal corticosteroids (Skoner 2001). Treatment of allergic rhinitis with topical glucocorticosteroids also improves the outcomes of asthma (Stelmach et al. 2005, Taramarcaz & Gibson 2004). Leukotriene receptor antagonists can be useful alone for mild asthma or allergic rhinitis, or in combination with glucocorticosteroids for more severe disease. Evidence is increasing that systemic treatment of asthma or allergic rhinitis with leukotriene receptor antagonists benefits both the upper and lower airways by improving symptoms and signs of inflammation (Jeffery & Haahtela 2006). Three-year subcutaneous specific immunotherapy in children has shown good efficacy in long-term control of allergic rhinitis and prevention of the development of asthma (Jacobsen et al. 2007).

Bronchial hyper-responsiveness and eosinophilic airway inflammation

Increased bronchial hyper-responsiveness (BHR) is one of the main pathophysiological changes in asthma. It is thought to result from airway inflammation, and in chronic disease, to be partly caused by smooth muscle hypertrophy and other structural changes in the bronchial walls (Bardana 2002). BHR can be found in 18 to 70% of patients with AD, even without a diagnosis of asthma, depending on the method used and the patient age (Crockcroft et al. 1984, Cookson et al. 1986, Backer et al. 1992, Brinkman et al. 1997). BHR, usually mild, can be found in 10 to 20% of healthy asymptomatic individuals (Sovijärvi et al. 1993, Bardana 2002). BHR can also be observed in patients with COPD and in up to 50 to 70% of patients with allergic rhinitis (Bardana 2002, Corren 2007).

Moderate or severe BHR is typical for asthma (GINA). Some fluctuation in BHR over time is possible, for instance due to respiratory tract infections or seasonal allergies (Bardana 2002). In population-based studies, bronchial responsiveness in adults is usually stable and is more likely to increase—especially in association with smoking—than to decrease (Chinn et al. 2005). Overall improvement in the atopic constitution may occur during adolescence, and BHR often diminishes along with the linear growth in adolescence (Ulrik et al. 1998).

Another of the main pathophysiological changes in asthma is chronic airway inflammation with eosinophils. Predominantly, eosinophilic airway inflammation occurs not only in asthma but in other airway diseases such as chronic cough, episodic symptoms without asthma (asthma-like inflammation), allergic rhinitis, and COPD (Gibson et al. 2002, Ryttilä et al. 2000). Smoking is associated with increased BHR and increased eosinophils in the sputum (Petäys et al. 2003).

“Atopic march”

Patients with AD often develop other atopy-related diseases or have family members suffering from them. Typically AD precedes the development of asthma, usually in

early childhood, and is accompanied by asthma and rhinitis later at preschool or school age or in early adulthood. The development of these atopic diseases with age is often called “the atopic march” (Spergel & Paller 2003). Risk factors for allergic asthma include positive family history for asthma and atopic diseases, allergic sensitization, allergic rhinitis, and AD, especially severe AD (Arruda et al. 2005, Corren 2007). According to several studies on the epidemiology of the atopic diseases, 40 to 50% of AD patients will develop asthma and approximately the same percentage allergic rhinitis (Spergel 2005, Corren 2007). Co-morbid AD and allergic rhinitis further increase asthma risk (Arruda et al. 2005). In patients with severe AD, occurrence of asthma may be over 60%. (Spergel 2005, Gustafsson et al. 2000). In one study, only 16% of AD patients developed neither asthma nor allergic rhinitis (Spergel 2005).

Several studies suggest a connection between the skin and airways and that epicutaneous antigen exposure may promote systemic allergic responses. Epicutaneous exposure to peanut via barrier-disrupted skin has led to specific IgE-production and Th2 type immune responses (Lack et al. 2003, Strid et al. 2005). A mouse model showed that epicutaneous sensitization to ovalbumin may be the first step leading to bronchial hyper-responsiveness (Spergel et al. 1998). Similarly, in mice, epicutaneous latex exposure can induce lung inflammation and airway hyper-responsiveness (Lehto et al. 2005), and epicutaneous *Aspergillus fumigatus* exposure may result in systemic Th2 immunity predisposing to allergic nasal responses (Akei et al. 2006).

Several reviews have concluded that skin barrier dysfunction correlates directly with both the AD severity and the probability of progression through atopic march to asthma; such dysfunction thus may be the initiating factor in the “atopic march” (Spergel & Paller 2003, Beck & Leung 2000). The fact that many genes related to AD are expressed in the epidermis or mucosa suggests that epithelial surfaces may initiate the atopic disease process (Cookson 2004). The actual mechanism behind the sequential development of atopic diseases may be in the AD-related systemic T cell trafficking from the skin to distant lymph nodes where Th2 type cytokines direct naive T cells to develop into Th2 type cells. These cells will then migrate to other tissues such as the bronchial and nasal mucosa where they start the atopic inflammatory process (Spergel & Paller 2003).

Atopic blepharoconjunctivitis

Allergic conjunctivitis is often associated with allergic rhinitis, whereas atopic blepharoconjunctivitis—or atopic keratoconjunctivitis—affecting also the eyelids and possibly the cornea is a chronic ocular manifestation of AD (Foster et al. 1991). It usually develops in middle-age, and most of the patients have AD in other skin areas, usually the face and neck (Foster et al. 1991). The histopathology of atopic blepharoconjunctivitis shares features of both acute allergic conjunctivitis, and chronic atopic inflammation. Eosinophils, mast cells, neutrophils, and lymphocytes, especially activated T cells, are present in conjunctival cytology (Bielory 2002). The primary

symptom of atopic blepharoconjunctivitis is intense bilateral itching of the lid skin, periorbital area, and conjunctiva. Tearing, burning, photophobia, blurred vision, and a stringy, rope-like mucus discharge also occur. The eyelids tend to be thickened, indurated, erythematous, fissured, and frequently swollen. The conjunctiva can be hyperemic and edematous, and tarsal conjunctival papillae are common. The patients often have dry eyes due to Meibomian gland dysfunction (Bielory 2002). Staphylococcal infections are common. The inflammation may also affect the cornea as punctate keratitis or as persistent epithelial defects. Corneal ulceration, recurrent severe *Herpes Simplex* keratitis, and formation of keratoconus are more severe consequences. If untreated, the disease may be sight-threatening (Bielory 2002). Treatment based on eye-drops containing antihistamines or sodium chromoglycate and its derivatives is often insufficient, with addition of glucocorticoids therefore often required. However, risk for glaucoma, cataract, HSV keratitis, and atrophy of the eyelid skin limits use of glucocorticoids to short periods, often resulting in an inadequate long-term treatment response (Clark 1995). Ocular *ex tempore* formulations containing cyclosporine are sometimes used for persistent disease (Zhan et al. 2003, Ono & Abelson 2005).

Conventional treatment of atopic dermatitis

Topical corticosteroids

Glucocorticosteroids are widely used in many inflammatory chronic diseases such as skin diseases, asthma, and rheumatic and connective tissue diseases. Topical glucocorticosteroids (corticosteroids) have been the mainstay of the AD treatment for the last few decades. They mediate immunosuppressive and anti-inflammatory effects via binding to specific receptors belonging to the steroid hormone superfamily and have been found in most tissues, including fibroblasts and osteocytes. Their most important anti-inflammatory effects are inhibition of vasoactive substances such as kinins, histamine, prostaglandins, leukotriens, and complement. They reduce the permeability of cell membranes, and inhibit migration of leucocytes and macrophages, as well as inhibit serum extravasation and edema by reducing the permeability of the vascular endothelium. They are also antipruritic (Hughes & Rustin 1997).

Glucocorticosteroid molecules are relatively small and in topical use passively diffused transepidermally or transfollicularly through the skin. Their absorption varies greatly depending on skin area, skin moisture balance, and temperature. Absorption through thin skin areas, such as eyelid or scrotum skin, is dozens of times higher than for thick skin areas, such as the scalp, palms, or soles. The infant skin is highly more permeable to corticosteroids than older children's or adults' skin (Goa 1988).

Corticosteroids can be classified according to their potency, which is determined by their vasoconstrictor properties. Hydrocortisone or cortisol is the most important

naturally occurring glucocorticosteroid secreted by the cortex of the adrenal glands; it alone comprises the group of mild topical corticosteroids. It is generally used for mild dermatitis or on thin skin areas such as the face, neck, eyelids, intertriginous areas, and scrotum. The group of midpotent corticosteroids sold in Finland include synthetic hydrocortisone 17-butyrate, desonide, and clobetasone butyrate. These may be indicated for mild to moderate eczema. Desoxymetasone and methylprednisolone aceponate, with the halogenated corticosteroid compounds betametasone 17-valerate and mometasone furoate, form the group of potent corticosteroids that are indicated for such conditions as moderate-to-severe dermatitis and psoriasis. The very potent clobetasone propionate and betametasone dipropionate are indicated for resistant severe dermatoses and for thick skin areas. (Lehmuskallio et al. 1998)

Cutaneous side-effects of topical corticosteroids include skin atrophy, bruising, telangiectasies, striae, steroid acne, hypertrichosis, tachyphylaxis, increased treatment tolerance (steroid resistance), and worsening of underlying secondary infections (Smith 1976). Topical corticosteroids for treatment of eyelid and facial eczema may cause elevation of intraocular pressure and induce glaucoma and cataracts (Clark 1995). These problems are common in long-term use of the compounds.

Topical corticosteroids are officially indicated only for short-term use and are usually used for only 1 to 3 weeks. They relieve the symptoms and inflammation of AD quickly, but after the treatment period the disease is likely to relapse (Lehmuskallio et al. 1998).

Secondary treatments

Secondary treatments of AD are combined with topical corticosteroids when these are not sufficient in control of the disease. They may diminish the need for topical corticosteroids but do not replace them.

UV-treatments

Several types of ultraviolet (UV) light therapy exist: UVB (wavelength 280-320 nm), narrow-band UVB (311-313 nm), UVA (320-400 nm), UVA1 (>340 nm), combined UVA-UVB also called selective ultraviolet phototherapy (SUP), and PUVA (UVA with usually topical photosensitizing psoralens). UVB induces immunosuppressive T regulatory cells through diminished antigen presentation of UV-damaged LCs and affects the cytokine production of keratinocytes (Schwartz 2005). UVA also alters LC functions and has effects on eosinophils (Krutmann & Morita 2002). UV-light treatment is given as 10- to 20-time serial treatments and is suitable for patients, whose AD improves during sunlight exposure in the summer time, but not for UV-sensitive patients. Most common is SUP, which has an efficacy similar to that of UVB (Jekler 1992). Narrow-band UVB treatment is growing more common and has shown superior efficacy to that of SUP (George et al. 1993, Gambichler et al. 2005) and similar efficacy to bath-PUVA (Der-Petrossian et al. 2000). UV light treatment is not usually suitable

for the acute phase of AD, except for UVA1, but serves as concomitant therapy with topical treatment or as maintenance therapy (Meduri et al. 2007). The length of the remission after the treatment period varies greatly and is longer for narrow-band UVB than for UVA (Meduri et al. 2007). Adverse effects include skin erythema, which is less with UVB treatment, skin burning, and risk for skin malignancies with cumulative UV dose (Krutmann & Morita 2002).

Antihistamines

Antihistamines are widely used for patients with atopic dermatitis, especially for pruritus, and also for co-existent allergic rhinoconjunctivitis and allergic asthma. First-generation compounds such as hydroxyzine are useful for nocturnal pruritus due to their sedative effect. Second- and third- generation antihistamines, such as cetirizine, levocetirizine, loratadine, desloratadine, and ebastine are effective against pruritic immediate allergic reactions and urticaria. These compounds have effects on many mediators of inflammation and can be assumed to exert effects in the acute phase of AD (Hoare et al. 2000). An evidence-based review, however, failed to find sufficient evidence that they relieve AD itself or the itching caused by AD (Klein & Clark 1999). The ETAC study (Early Treatment of the Atopic Child) compared cetirizine with placebo. Cetirizine reduced the need for potent corticosteroids in patients with more severe AD, but had no effect on AD in general, because the use of topical corticosteroids and decrease in severity parameters in the cetirizine and placebo groups were similar (Diepgen et al. 2002).

Systemic glucocorticosteroids

Systemic glucocorticosteroids such as prednisone, prednisolone, and methylprednisolone often serve as rescue therapy in severe AD exacerbations. They relieve itch and inflammation quickly, but the risk for AD relapse soon after cessation of treatment is high. In addition to having the same side-effects as do topical corticosteroids, they may cause arterial hypertension, electrolyte imbalance, impaired glucose metabolism, Cushing's syndrome, and osteoporosis, especially in long-term treatment (Hoare et al. 2000).

Ciclosporin

Severe AD that does not respond adequately to topical treatment or combined topical and UV-light treatment often requires the addition of systemic long-term immunosuppressive treatment. However, these treatments will diminish but not abolish the need for topical treatments. Ciclosporin (cyclosporin A) is a nonmyelosuppressive immunosuppressant effective in cell-mediated immune responses and widely used in organ transplant patients (Granlund et al. 1995). It is also used in a variety of autoimmune diseases such as rheumatoid diseases, psoriatic arthritis, severe psoriasis,

severe atopic dermatitis, hand eczema, and pustulosis palmoplantaris (Aberer & Wolff 2002). Ciclosporin for AD is extensively studied and has shown good efficacy in adults and children (Sowden et al. 1991, van Joost et al. 1994, Granlund et al. 1995, Zonneweld et al. 1996, Berth-Jones et al. 1997, Harper et al. 2000). It acts by down-regulating and inhibiting the cytokine production of Th2 cells. (Granlund et al. 1995) Treatment of AD with ciclosporin requires a regular follow-up by a dermatologist due to its possible adverse effects: arterial hypertension, nephrotoxicity, and immunosuppression (Aberer & Wolff 2002). The usual initial dose is 2.0 to 5.0 mg/kg, which should be tapered down to the lowest effective dose (Aberer & Wolff 2002). Treatment recommendations are different. Some clinicians favor short-term cycles to avoid adverse effects, some continuous treatment for up to one year to avoid relapses, and some long-term intermittent treatment (Aberer & Wolff 2002, Granlund et al. 1995, Schmitt et al. 2007).

Azathioprine and methotrexate

Azathioprine is an old compound used for similar indications as for cyclosporine. Its precise mode of action in AD is unknown (Berth-Jones et al. 2002), but it has been used for severe AD especially in the United Kingdom. Until lately, randomized, controlled studies on its efficacy and safety for AD have been few. In two 12-week studies it caused a mean reduction in disease activity of 27% in severe (Berth-Jones et al. 2002) and 37% in moderate-to-severe (Meggitt et al. 2006) AD. Adverse effects include bone marrow suppression and oncogenic potential (Schmitt et al. 2007). At present, the long-term effects of azathioprine in AD are unknown.

Methotrexate is another old drug compound used similarly to azathioprine. At present, in addition to case reports and retrospective studies, only one prospective study examines its use in AD. In this uncontrolled 24-week study of 12 patients with severe AD unresponsive to topical corticosteroids, methotrexate reduced the disease activity by a mean of 52% (Weatherhead et al. 2007). Its main adverse effects include liver toxicity, anemia, thrombopenia, gastrointestinal dysfunction, and pneumonitis (Schmitt et al. 2007).

Others

Other newer treatment modalities tried for severe treatment-resistant AD include mycophenolate mofetil, interferon gamma (IFN- γ), intravenous immunoglobulin (IVIG), and the new TNF- α inhibitors or “biologic” treatments designed for rheumatoid arthritis and psoriasis. Mycophenolate mofetil has shown a promising response in two small open, uncontrolled studies and one retrospective study with treatment responses of 55 to 85% (Murray & Cohen 2007). Recombinant IFN- γ treatment has shown variable results (Schmitt et al. 2007). Results with IVIG have been disappointing (Bemania et al. 2005, Schmitt et al. 2007). At present, the TNF- α inhibitors for AD have been addressed only in case reports or small pilot studies. Some efficacy has resulted from efalizumab and

infliximab but not from etanercept (Takiguchi et al. 2007, Jacobi et al. 2005, Buka et al. 2005).

Leukotriene receptor antagonists have shown efficacy in the treatment of asthma and allergic rhinitis (Busse & Kraft 2005). In AD, they have been efficacious as steroid-sparing agents in case studies, but in randomized controlled studies the results have been controversial, and they have shown no efficacy in severe AD (Veien et al. 2005, Silverberg & Paller 2004).

Mepolizumab, anti-IL-5 recombinant humanized monoclonal antibody that inhibits eosinophil functions, reduced peripheral blood eosinophils but failed to show efficacy in AD (Oldhoff et al. 2005). Anti-immunoglobulin E (omalizumab), which binds to free IgE and membrane-bound IgE on B cells, has shown efficacy in allergic respiratory disease (Chang et al. 2007). Only case reports exist on its efficacy on AD (Lane et al. 2007, Forman & Garret 2007), but larger studies are lacking.

Topical calcineurin inhibitors

Tacrolimus ointment – Preclinical studies

The tacrolimus (FK506) molecule has a macrolide lactone structure and a molecular weight of 822 Da. It was isolated from a strain of soil bacteria called *Streptomyces tsukubaensis* on Mount Tsukuba, Japan, in 1984, and found to have a strong immunosuppressive effect on lymphocytes without cell damage (Kino et al. 1987). In the following *in vitro* studies its potency turned out to be dramatically greater than that of ciclosporin and prednisolone, and it inhibited the production of the T cell activator IL-2, and expression of the receptor for IL-2, as well as IL-3 and IFN- γ (Goto & Nagakawa 2004). The immunosuppressive action of tacrolimus was found specific to T cells as it did not inhibit B cells, natural killer cells, or various bone marrow-derived cell lines. That prednisolone was ten times and ciclosporin twice as bone marrow-suppressive as tacrolimus suggested that tacrolimus is an effective non-toxic immunomodulatory agent (Goto & Nagakawa 2004). Despite its macrolide structure, tacrolimus has failed to show marked antimicrobial activity except on the fungi *Fusarium oxysporum*, *Aspergillus fumigatus*, and *Malassezia furfur* (Nagakawa et al. 1996). Its potent immunomodulatory effect in humoral and cell mediated immune reactions has been demonstrated in several experiments in mice, in which oral tacrolimus is several times more potent than ciclosporin (Goto & Nagakawa 2004). These results have been repeated in numerous murine models of experimental autoimmune diseases such as arthritis, systemic lupus erythematosus, allergic encephalitis, and uveitis, as well as graft-versus-host disease (Goto & Nagakawa 2004).

The mechanism of action of tacrolimus is its inhibition of the calcineurin pathway. Tacrolimus binds to a macrophilin called FK506-binding protein (FKBP-12),

and their complex inhibits activation of NF-AT, which leads to inhibition of T cell activation and proliferation by down-regulating the IL-2 gene (Dumont 2000). It also inhibits production of several proinflammatory cytokines such as IL-3, IL-4, IL-5, IFN- γ , tumor necrosis factor- α , and granulocyte-macrophage colony-stimulating factor (Wollenberg et al. 2001). Tacrolimus reduces the proportion of IDECs in the epidermis of AD skin and down-regulates the expression of Fc ϵ RI, thus weakening the antigen-presenting capacity of these cells (Schuller et al. 2004, Wollenberg et al. 2001). Tacrolimus causes increased expression of TGF- β and decreased expression of tumor necrosis factor- α and nitric oxide synthase in keratinocytes (Lan et al. 2004, Lan et al. 2005). It also suppresses the secretion of cytokines and other mediators from eosinophils and basophils (Kohyama et al. 1999, Sengoku et al. 2000, Plath et al. 2003, Simon et al. 2004).

Systemic tacrolimus was launched first in 1993 in Japan and in 1994 in the USA and UK for solid organ transplant rejection and the prevention and treatment of graft-versus-host reactions after bone marrow transplantation. Development of topical tacrolimus was started after transplant patients receiving tacrolimus showed improvement in their co-existent AD or psoriasis.

Topical tacrolimus was first tested in animal models. In NC/Nga mice it inhibited the spontaneous dermatitis and was effective against established dermatitis by suppressing T cells, eosinophils, masT cells, IL-4, IL-5, and IgE. It also inhibited delayed hypersensitivity reactions in different models of experimental contact dermatitis or atopic dermatitis. (Goto & Nakagawa 2004) An ointment formulation was chosen as the vehicle due to the high lipophilicity of the tacrolimus molecule. Concentrations of 0.1% and 0.3% showed equal efficacy to 0.5% and 1.0% concentrations in preventing dermatitis reactions in mice and were chosen for further clinical investigation. The first clinical studies of topical tacrolimus in chronic plaque psoriasis showed some efficacy only under occlusion (Zonneveld et al. 1998, Remitz et al. 1999), so developmental work was focused on AD.

Tacrolimus ointment - Clinical studies

Efficacy

Short-term studies

In several vehicle-controlled short-term studies, 0.03%, 0.1%, and 0.3% tacrolimus ointments were significantly more effective than the vehicle in AD (Reitamo et al. 2002) in adults and children. No significant differences emerged between different concentrations of tacrolimus. Tacrolimus 0.03% and 0.1% ointments were chosen for corticosteroid-controlled studies. In a randomized, double-blind study in 2- to 15-year-old children with moderate-to-severe AD, both concentrations were significantly superior to 1% hydrocortisone acetate (Reitamo et al. 2002). In adult patients with moderate-to-severe AD, tacrolimus 0.1% showed similar efficacy as hydrocortisone 17-

butyrate, and both were superior to the 0.03% ointment (Reitamo et al. 2002). In a pediatric 3-week study, tacrolimus 0.03% ointment applied once or twice daily was more efficient than hydrocortisone 1% acetate twice daily. Tacrolimus ointment twice daily was more effective than once daily, especially in patients with more severe baseline disease (Reitamo et al. 2004). A 6-week study showed the better efficacy of 0.1% ointment than of oral ciclosporin 3 mg/kg (Pacor et al. 2004).

Long-term studies

Long-term open-label, non-comparative, multicenter studies lasting for 12 to 48 months in Europe and the US have addressed the safety and efficacy of tacrolimus ointment in several thousand adult and pediatric patients. Both adult and pediatric patients with moderate-to severe AD treated with 0.1% tacrolimus ointment have showed rapid and sustained improvement in efficacy parameters (Reitamo et al. 2000, Kang et al. 2001, Hanifin et al. 2005). Pediatric patients treated with 0.03% ointment intermittently for 12 to 29 months and with 0.1% ointment periodically if the 0.03% was insufficient, showed substantial improvement in efficacy parameters including pruritus within 2 weeks maintaining the result throughout the study (Remitz et al. 2007).

Tacrolimus ointment 0.1% was compared with hydrocortisone 17-butyrate on the trunk and extremities and with 1% hydrocortisone acetate on the face and neck in a multicenter, randomized, double-blind, controlled study of 6 months in adult patients with moderate-to-severe AD. Tacrolimus provided greater improvement in all efficacy parameters, and a higher percentage of the patients showed at least a 60% response (72.6% vs. 52.3%, $p < 0.001$) (Reitamo et al. 2005).

Safety

Tacrolimus ointment has been safe in short- and especially long-term studies. No significant changes have occurred in laboratory parameters such as renal and liver function or hematology nor any severe adverse events related to the compound. Clinically, the most relevant adverse effect has been the burning sensation and pruritus of the treated skin during the first days of treatment (Hanifin et al. 2005, Reitamo et al. 2005, Remitz et al. 2007). The occurrence of skin infections (*S. aureus*, HSV, eczema herpeticum, fungal dermatitis, warts, mollusca) has been generally low, similar to, or lower than in previous reports on corticosteroid-treated patients, and has not increased with duration of exposure to tacrolimus ointment (Hanifin et al. 2005, Remitz et al. 2007). In the corticosteroid controlled long-term study, no increase occurred in incidence of infections over time in either treatment group (Reitamo et al. 2005).

Systemic exposure to tacrolimus from topical treatment has been low (Paller et al. 2001, Soter et al. 2001). The highest blood concentrations after topical treatment have been approximately 3% of those of transplant patients using systemic tacrolimus, with no signs of accumulation of tacrolimus after repeated application. Both the rate and

extent of topical absorption decrease as skin lesions heal (Rubins et al. 2005). These findings suggest that tacrolimus ointment is safe in long-term treatment.

In systemic use, tacrolimus is immunosuppressive and possibly associated with increased cancer, especially lymphoma, risk. Topical tacrolimus associated with non-melanoma skin cancer in a mouse model (Niwa et al. 2003). In the USA, this led to black box warnings on increased cancer risk from topical tacrolimus and pimecrolimus. However, a recent large case-control study with a cohort of over 290 000 patients receiving different AD treatments found no increased risk for lymphoma in patients treated with topical calcineurin inhibitors, the main factor associated with increased lymphoma risk being AD severity (Arellano et al. 2006). According to a recent systemic review on topical treatment of AD, significant local or systemic adverse events are mainly associated with use of topical corticosteroids (Callen et al. 2007).

Tacrolimus ointment - Monotherapy and maintenance therapy

Tacrolimus ointment is currently indicated for moderate-to-severe AD as an intermittent treatment. Results of intermittent or continuous monotherapy in long-term studies have been good (Reitamo et al. 2000, Kang et al. 2001, Hanifin et al. 2005, Remitz et al. 2007). Because tacrolimus is a relatively new compound and more expensive than topical corticosteroids, attempts have been made to combine it with topical corticosteroids. One study suggested that when tacrolimus ointment is used only on facial AD, and the other AD areas are treated with corticosteroids, the long-term results are poor despite a favorable initial response (Sugiura et al. 2000). These results correlated inversely with the AD severity and amount of corticosteroids. Recent as-yet unpublished studies in adults and children on maintenance therapy with tacrolimus ointment twice weekly after an initial treatment period twice daily for up to 6 weeks suggest that long-term control of AD can be achieved with fewer relapses and with reasonable use of tacrolimus ointment (EADV congress 2007, Rhodes). Similar results with potent corticosteroid compounds are published (Berth-Jones et al. 2003, Faergemann et al. 2000, Hanifin et al. 2002). Comparative studies with tacrolimus and corticosteroids in maintenance therapy are thus far lacking.

Pimecrolimus cream

Pimecrolimus is the other topical calcineurin inhibitor developed during the same period as tacrolimus. Pimecrolimus 1% cream is licenced for mild to moderate AD in adults and children. The drug molecule is very similar to tacrolimus but somewhat more lipophilic. It has an affinity one- third of that of tacrolimus to their common carrier protein FKBP12. Pimecrolimus cream has shown superior efficacy to its vehicle but is weaker than betamethasone (Luger et al. 2001). A long-term, randomized, controlled study with tricamcinolone acetate on the body and extremities, with hydrocortisone

acetate on the face and neck versus pimecrolimus, reported equal efficacy compared to that of the corticosteroid arm. However, no intention-to-treat analysis was done, and the discontinuation rate in the pimecrolimus group was 60% (Luger et al. 2004). In studies comparing tacrolimus and pimecrolimus, tacrolimus has shown superior efficacy with a similar adverse-effect profile (Paller et al. 2005). According to flare control studies, pimecrolimus cream seems to work best as a corticosteroid-sparing agent (Kapp et al. 2002, Wahn et al. 2002, Meurer et al. 2002 and 2004, Papp et al. 2005, Siegfried et al. 2006).

Evidence-based treatment of AD

AD has undergone various treatments for decades, although evidence-based data on these treatment modalities is scarce. Evidence-based medicine means treatment based on sufficient information from high-quality trials. Quality requirements include a well-defined and sufficiently large patient population and well-defined diagnostic methods and assessments of AD severity and treatment efficacy. Studies should also reflect clinical need for treatment so that chronic diseases are investigated in long-term studies. Studies on treatment of AD have usually been small in patient number, seldom controlled, or controlled with a poorly validated compound (Hoare et al. 2000).

Topical corticosteroids have been studied mostly in short-term trials, because no long-term studies have been required by authorities. The quality of the studies varies, and there are no published placebo-controlled trials for instance of betamethasone 17-valerate, considered a standard comparator for newer topical treatments. However, the chronic nature of AD calls for long-term controlled trials to find a safe and efficient way to control the disease. Recently three studies have lasted for 4 to 6 months testing maintenance therapy for AD with topical corticosteroids twice weekly with favorable results (Faergemann 2000, Berth-Jones et al. 2003, Hanifin et al. 2002). Intermittent long-term corticosteroid treatment has been used in two randomized controlled studies lasting for 6 months: one with tacrolimus ointment and one with pimecrolimus cream (Reitamo et al. 2005, Luger et al. 2004). At present, these are the only published corticosteroid controlled long-term studies on the AD treatment.

Data on the placebo-controlled short-term studies and controlled long-term studies of the most common treatments of AD are presented in Table 1.

In chronic diseases that can severely impair quality of life, such as AD, placebo-controlled long-term studies without a possibility for rescue therapy are not ethically acceptable for patients with moderate-to-severe disease. In randomized controlled studies, the selection criteria for eligible patients are strict, which leads to high exclusion numbers, and the final study population will then represent only a few types of the disease. For this reason, generalization of the results to population level is problematic, as patients in “real life” present with different types and variable degrees of the disease. In the recent years, long-term studies in “real-life” settings, where effects

of one type of treatment can be better extended to clinical practice, have become necessary for chronic diseases such as asthma (Bjermer 2006). At present, no such studies have appeared on AD.

Table 1. Studies on the most common treatments of AD including only those with efficacy >50%.

Treatment	Efficacy in placebo-controlled short-term studies (< 6 months)	Efficacy in controlled long-term studies (≥ 6 months)
Topical steroids	Betamethasone dipropionate (Vanderploeg 1976) Clobetasol propionate (Maloney et al. 1998) Desonide (Stalder et al. 1994) Halcinonide (Lupton et al. 1982) Hydrocortisone buteprate (Sears et al. 1997) Hydrocortisone valerate (Sefton et al. 1984) Fluticasone propionate (Lebwohl et al. 1996) Fluticasone propionate in maintenance therapy twice weekly (Berth-Jones et al. 2003)	Fluticasone propionate vs. vehicle in maintenance therapy twice weekly (Hanifin et al. 2002)
UV-light treatments	Broad band UVB (Jekler & Larkö 1988) Narrow band UVB, UVA (Reynolds et al. 2001)	Unavailable
Ciclosporin	5 mg/kg (Munro et al. 1994, Salek et al. 1993, Sowden et al. 1991, van Joost et al. 1994, Wahlgren et al. 1990)	3 mg/kg vs. 5mg/kg (Zonneveld et al. 1996) Short courses vs. continuous (Harper et al. 2000)
Systemic steroids	Unavailable	Unavailable
Methotrexate	Unavailable	Unavailable
Azathioprine	Unavailable	Unavailable
Tacrolimus ointment	0.03% in children (Schachner et al. 2005) 0.03% in adults and children (Chapman et al. 2005) 0.1% and 0.03% in adults and children (Hanifin et al. 2001, Paller et al. 2001) 0.03%, 0.1%, and 0.3% in adults and children (Ruzicka et al. 1997, Boguniewicz et al. 1998)	0.1% tacrolimus vs. hydrocortisone 17-butyrate/ hydrocortisone (Reitamo et al. 2005)
Pimecrolimus cream	Adults (Eichenfield et al. 2002) Children (Ho et al. 2003)	Pimecrolimus vs. vehicle in adults, flare control (Meurer et al. 2002, 2004) Pimecrolimus vs. vehicle in children, flare control (Wahn et al. 2002, Kapp et al. 2002, Papp et al. 2004) Pimecrolimus vs. triamcinolone acetate / hydrocortisone (Luger et al. 2004) (No intention-to-treat analysis)

Specific issues related to treatment of atopic dermatitis

Effects of treatment on skin barrier function

Topical corticosteroids are known to induce stratum corneum thinning. They cause upregulation of endogenous proteases that cause degradation of corneodesmosomes forming junctions between corneocytes. These changes, leading to clinically measurable increase in TEWL, can already be detected already after short (3-7 days) treatment periods as well as after longer-term treatment (Cork et al. 2006). Treatment of AD skin with tacrolimus ointment reduces TEWL to normal or almost normal levels, which suggests that tacrolimus could have a normalizing effect on skin barrier function (Granlund et al. 2001, Pournaras et al. 2002).

Skin atrophy and collagen synthesis of the skin

A well known problem related to topical corticosteroid treatment is skin atrophy. Naturally thin skin areas such as the face, neck, eyelids, and scrotum are especially sensitive for the atrophogenic effect. Skin atrophy is caused by suppression of connective tissue, especially collagen synthesis in the dermal fibroblasts. Long-term treatment or repeated treatment periods lead to clinically visible skin changes: redness, telangiectasies, striae, bruising, and thinning (Smith et al. 1976). At laboratory level, however, findings suggesting suppressed collagen synthesis can be seen even after short treatment periods. Methods suitable for assessing skin atrophy noninvasively include skin ultrasound for measuring skin thickness, and collection of dermal suction blister fluid (SBF) from which procollagen propeptides can be measured (Kiistala 1968). Collection and analysis of suction blister fluid reflects skin collagen synthesis specifically and is unaffected by synthesis from other organs unlike the corresponding markers detected in serum (Oikarinen et al. 1992). In ultrasound, atrophy can be masked by inflammation and edema, as seen in atopic dermatitis, which can result in increased skin thickness despite decreased collagen synthesis (Reitamo et al. 1998). Other factors such as age, sex, time of day, and hormonal phase in women may also have an effect on skin thickness (Eisenbeiss et al. 1998 and 2001, Gniadecka et al. 1994, Seidenari et al. 1994, Haapasaari et al. 1996).

Collagen synthesis of the skin

Collagens are the most abundant protein of human connective tissue and can be found in organs and structures requiring tensile strength. Collagen fibers make up 70 to 80% of the dry weight of the skin and tendons, 50 to 60% of cartilage, and about 90% of the organic tissue of mineralized bone, and occur also in the walls of the great arteries and

liver (Burgeson & Nimni 1992). At least 19 different types of collagen exist in general, and five different types of collagen are found in the skin, the major types being I and III. Type I is also found in bone and type III in soft tissues. Collagen type IV structures the basement membranes and type VII the anchoring fibrils. (Burgeson & Nimni 1992) Collagen fibers consist of bundles of collagen fibrils. The collagen fibrils consist of collagen molecules in which three α -chains form a triple-helical structure. Every third aminoacid in the collagen molecule is glycine. Hydroxylysine, 4-hydroxyproline, and proline are the other most important aminoacids (Prockop et al. 1979, Burgeson & Nimni 1992).

The interstitial collagen molecule is synthesized in the endoplasmic reticulum of fibroblasts in the skin and other relevant cells in other tissues in a precursor form called procollagen. Procollagen consists of three pro α -chains which are longer than the mature collagen due to procollagen propeptides at their amino- and carboxy-terminal ends. Several crucial enzymatic steps are necessary in formation of the collagen fibril and its secretion into the extracellular matrix. Finally the amino- and carboxy-terminal procollagen propeptides are cleaved off as one block, and the remaining mature collagen fibrils then undergo spontaneous assembly to functional collagen fibers. Bivalent cross-links are formed between these collagen fibers. Later some of these cross-links will develop into multivalent cross-links, which makes the collagen fiber very stable and resistant to proteolytic enzymes (Prockop et al. 1979, Burgeson & Nimni 1992). The collagen bundles can consist of one or several types of collagen fibers. Type I and type III collagen fibers are known to make copolymers, in which type III fiber coats the surface of the type I collagen fiber (Fleischmajer et al. 1990).

The aminoterminal procollagen propeptide of collagens type I and type III are called PINP and PIIINP, and the carboxy-terminal procollagen propeptide of collagen type I is called PICP. The cleaved procollagen propeptides are soluble and can be measured in blood or in suction blister fluid obtained from the skin to estimate collagen synthesis in vivo (Oikarinen et al. 1998). PIIINP and PICP cleavages occur equally suggesting that the synthesis of type I and III collagens is coordinated (Autio et al. 1993).

Collagen degradation is a controlled process that enables appropriate extracellular matrix remodelling to occur. Excess collagen molecules are removed by degrading enzymes, collagenases, which belong to the family of matrix metalloproteinases (MMPs). Collagens I and III are degraded by interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). The proteolytic activity of MMPs is in turn inhibited by tissue inhibitors of metalloproteinases (TIMP) (Haapasaari 1997).

Effects of glucocorticosteroids on skin collagen synthesis

Corticosteroids reduce the synthesis of collagen types I and III, and glycosaminoglycans (GAG) (Oikarinen et al. 1992), which leads to reduction in water content and in overall

skin thickness (Sarnstrand et al. 1982). Corticosteroids modify gene transcription by interacting with glucocorticoid response elements (GREs), which are present in the promoter regions of a variety of genes found in many cell types (Scheinman et al. 1995). This broad mode of action translates into a range of effects, including suppression of dermal fibroblast function and proliferation. The direct effect on fibroblasts leads to reduction in the synthesis of collagen and of GAG and to a modification in the composition of GAGs, ultimately resulting in skin atrophy (Hughes & Rustin 1997) (Table 2).

All topical corticosteroid groups of differing potency have suppressed collagen synthesis in short-term studies performed in healthy subjects. The changes are already evident after short treatment periods lasting for 3 to 7 days (Haapasaari et al. 1995, Koivukangas et al. 1995, Oikarinen et al. 1998). In a one-week study comparing glucocorticosteroids of differing potency, hydrocortisone reduced both PINP and PIIINP by a respective 35% and 35%, hydrocortisone butyrate by 63% and 55%, and betamethasone valerate by 69% and 62% (Haapasaari et al. 1995). In later studies, the suppressive effect on collagen synthesis of the mildest glucocorticosteroid hydrocortisone has been similar or nearly similar to that of the more potent compounds (Haapasaari et al. 1997, Nuutinen et al. 2003). The glucocorticoid-induced decrease in collagen propeptides is due to a corresponding decrease in functional collagen messenger RNA in vivo (Oikarinen et al. 1998). After a 3-day treatment with betamethasone valerate, recovery of collagen synthesis is only partial during a 2-week treatment-free period (Haapasaari et al. 1996). Hydrocortisone used intermittently as 3 application days weekly is less atrophogenic than is continuous treatment, and with both regimens, complete recovery of collagen synthesis is achieved after a 3-week treatment-free period (Nuutinen et al. 2003).

In a long-term study with inhaled corticosteroids for asthma for 1 to 2 years, some decrease in collagen synthesis in the skin and bone is evident, but on the other hand, the total amount of collagen in skin biopsies does not decrease significantly except in patients receiving additional peroral corticosteroids (Haapasaari et al. 1997); it can thus be speculated that in long-term glucocorticoid treatment collagen degradation may also slow down. An 8-day treatment of dorsal rat skin with subcutaneous dexamethasone reduces not only the synthesis of collagens I and III but also levels of collagenase and its messenger-RNA as well as levels of corresponding TIMPs (Oishi et al. 2002). These findings suggest that deterioration of skin function may be due to simultaneous suppression of MMPs such as collagenases.

In long-term maintenance therapy studies for AD with potent topical corticosteroids, atrophogenicity has been followed only subjectively; no marked signs of skin atrophy appeared (Faergemann et al. 2000, Berth-Jones et al. 2003, Hanifin et al. 2002). In a one-week study in AD patients and healthy controls, when measured by procollagen propeptide concentrations in SBF and compared with placebo, tacrolimus ointment showed no atrophogenic potential (Reitamo et al. 1998). Of other treatments,

UVA therapy suppresses collagen synthesis, whereas UVB treatment for one week does not (Mempel et al. 2000, Autio et al. 1994).

Table 2. Results of studies on collagen synthesis after systemic, inhaled, or topical corticosteroid treatment in subjects with healthy skin or dermatoses*†.

Study	Compound	Length	Method	Decrease (%) in		
				PINP	PIINP	PICP
Oikarinen et al. 1992*	Prednisone, Prednisolone, Methylprednisolone (systemic); N=22	5 ± ? Days	Serum		38	34
Autio et al. 1994**	Prednisone (systemic); N=10			14	13	18
Autio et al. 1996	Budesonide (inhaled) 400 µg/d; N=9	6 weeks	SBF	47	63	58
	Budesonide (inhaled) 1600 µg/d; N=9			39	51	41
Haapasaari et al. 1998	Budesonide 800-1600 µg/d; N=23 or Beclomethasone dipropionate 800-1000 µg/d (inhaled); N=4	6 months	SBF	70	76	
				13	20	0
Koivukangas et al. 1995	Betamethasone valerate; N =15	1 week	SBF	56	54	38
	Momethasone furoate; N=15		Serum	59	54	33
Haapasaari et al. 1995	Hydrocortisone; N=4	1 week	SBF	35	35	
	Hydrocortisone 17-butyrate; N=5			63	55	
	Betamethasone valerate; N=5			69	62	
Haapasaari et al. 1996	Betamethasone valerate; N=28	3 days	SBF	79-86	64-81	
Haapasaari et al. 1997	Hydrocortisone; N=15	1 week	SBF	66	62	
	Methylprednisolone aceponate; N=15			68	68	
	Momethasone furoate; N=15			72	72	
Oikarinen et al. 1998	Betamethasone valerate; N=10	3 days	SBF	82	73	
Nuutinen et al. 2003	Hydrocortisone 3 times/week; N=9	3 weeks	SBF	53	50	
	Hydrocortisone twice daily; N=9			89	82	
Reitamo et al. 1998 †	Betamethasone valerate;	1 week (under occlusion)	SBF	82	61	83
	Vehicle			0	0	0
	Tacrolimus 0.1%			0	0	0
	Tacrolimus 0.3%			0	0	0
	N=25 total in all groups					

* Patients were treated for different dermatoses, 5 for AD

** Relative proportion of values of the non-treated healthy controls after one measurement

† Healthy controls (n=12) and patients with AD (n=13)

PINP=Aminoterminal propeptide of procollagen type I

PIINP=Aminoterminal propeptide of procollagen type III

PICP=Carboxyterminal propeptide of procollagen type I

SBF=suction blister fluid

Effects of AD treatment on skin infections

Topical corticosteroids have reduced *S. aureus* colonization on AD skin in short-term studies lasting 1 to 8 weeks (Stalder et al. 1994, Hung et al. 2007, Gong et al. 2006). Tacrolimus 0.1% and 0.03% ointments have been effective in eradication of *S. aureus* colonization in short-term studies of 3 to 8 weeks (Pournaras et al. 2002, Park et al. 2005, Hung et al. 2007). Tacrolimus 0.03% was effective but slower in *S. aureus* eradication than was fluticasone 0.05% (Hung et al. 2007). Maintenance therapy of AD with mometasone furoate twice weekly for six months decreased *S. aureus* as well as *Malassezia* (*Pityrosporum*) colonization in cleared patients (Faergemann et al. 2000). UVB and PUVA, but not UVA alone, decrease *S. aureus* colonization and superantigens (Silva et al. 2006, Jekler et al. 1992, Yoshimura-Mishima et al. 1999). Ciclosporin has shown some effect on *S. aureus* colonization and superantigen when combined with topical betamethasone (Bunikowski et al. 2003).

Infected AD exacerbations require specific treatment of the microbe in combination with eczema treatment, but no evidence supports the assumption that antimicrobial treatment of colonized skin will benefit patients in the long-term. Combining topical antibiotic agents with anti-inflammatory treatment has led to no further decrease in *S. aureus* colonization (Hung et al. 2007, Gong et al. 2006, Williams 2000). Neither is there evidence that antifungal treatment's reducing *Malassezia* colonization would relieve AD in the long-term, although treatment periods with an antifungal agent have had some effect, especially on eczema of the sebaceous areas (Baker 2006).

Anti-inflammatory treatments (corticosteroids, calcineurin inhibitors) may trigger HSV infections (Novak & Bieber 2005). In a randomized controlled study of patients with moderate-to-severe AD, tacrolimus was compared with hydrocortisone-17-butyrate for 6 months. The prevalence of HSV infections during the first month of the study was higher in the tacrolimus group (2.9% vs. 1.0%), but the prevalence decreased to a level comparable to that of corticosteroids during the rest of the study months (1.3% vs. 1.0%) (Reitamo et al. 2005). In a similar study of 48 months, the prevalence of HSV in the tacrolimus group vanished during follow-up (Hanifin et al. 2005).

AIMS OF THE STUDY

Atopic dermatitis is a chronic pruritic skin disease often complicated by *Staphylococcus aureus* colonization and superinfection. In patients with AD, concomitant occurrence of other atopic diseases like asthma and allergic rhinoconjunctivitis is common. Skin atrophy is the major problem in conventional corticosteroid-based treatment of AD, which limits treatment to short periods, especially in thin skin areas. After the treatment period, AD often relapses. In practice, mainly the patients with mild disease achieve a longer remission with short-term use of topical corticosteroids. Patients with moderate or severe AD are usually poorly controlled with short-term treatments and experience continuous skin inflammation of varying degrees. The barrier function of the inflamed skin is weakened, possibly enabling an epicutaneous sensitization to occur which may lead to atopic respiratory disease. The need is therefore great for a non-atrophogenic treatment option, especially for patients with moderate-to-severe AD requiring long-term treatment.

Tacrolimus ointment, a safe and efficient topical treatment for AD, belongs to the group of topical immunomodulators which have a more specific mode of action on the skin than the broad-scale effects of topical corticosteroids. Based on a short-term study tacrolimus has shown no atrophogenic properties.

The general aim of this study was to evaluate the effects of tacrolimus ointment on clinically important problem areas related to AD with an emphasis on long-term AD treatment.

The specific aims of the study were to discover:

1. The effect of tacrolimus ointment on *Staphylococcus aureus* colonization in patients with moderate-to severe AD before and during treatment.
2. The characteristics of skin collagen synthesis and skin thickness in patients with moderate-to-severe AD before and after tacrolimus treatment.
3. Retrospectively, the efficacy of 0.03% tacrolimus ointment in treatment of atopic blepharoconjunctivitis and its effect on conjunctival inflammatory cells.
4. The prevalence of bronchial hyper-responsiveness, symptoms of asthma and allergic rhinitis, and sputum eosinophilia in patients with moderate-to-severe AD, and the effect of long-term treatment of AD with tacrolimus. The effect of long-term improvement in AD was indirectly evaluated by observation of the patients with the best long-term response.

SUBJECTS AND METHODS

Study designs

All studies were carried out in Skin and Allergy Hospital, Helsinki University Central Hospital, Helsinki, Finland, and were approved by the local ethics committee of Helsinki Central Hospital. All subjects gave their written informed consent before the start of the study. Use of tacrolimus ointment in Studies I, II, and V was permitted by the National Agency for Medicines (Lääkelaitos). Studies I, II, and V were single-center studies conducted in the context of prospective, multicenter, open-label, non-comparative, long-term safety studies in patients with moderate-to-severe AD using tacrolimus 0.1% ointment twice daily as an intermittent treatment. Study I included 6-month and 12-month populations. Study II lasted for 12 to 24 months, and Study V for 48 months. Study III was a cross-section study of AD patients and Study IV a retrospective study of patients with atopic blepharoconjunctivitis who were using 0.03% tacrolimus ointment.

Subjects

Male and female patients over 18 years of age were eligible for Studies I, II, and IV. Studies III and V included patients over age 13. For more characteristics, see Table 3.

Table 3. Characteristics of and reasons for withdrawal among study populations.

Subjects	N	Age	Gender: females	Length of follow-up	Discontinued (N): reasons
Study I	19 9	31.7 30.0	15 8	6 months 12 months	3: pregnancy, lack of efficacy, adverse event
Study II: Tacrolimus	56	26.8	35	12-24 months	
Corticosteroid	36	25.8	26	12 months	
Healthy	27	28.7	21		
Study III: AD patients	86	25.7	57		3: pregnancy, lack of efficacy, adverse event
Controls	49	26.9	13		
Study IV	10	33.0	6	6 (4-13) wk	
Study V	65	25.5	41	48 months	

Inclusion criteria

Patients with moderate-to-severe AD according to both Hanifin and Rajka (1980) and the Rajka and Langeland (1989) criteria were eligible for Studies I, II, III, and V. A body surface area (BSA) of AD of 5 to 60% was required for Study I and 5 to 100% for Studies II, III, and V. All the patients in Studies II and V had participated in previous tacrolimus ointment studies, one of 3 weeks' or 6 or 12 months' duration (Kang et al. 2001, Reitamo et al. 2002). Before entering the new study, all participants spent a tacrolimus-free interim period of 4 to 12 months in which treatment with topical corticosteroids was allowed. Study IV included patients who had been treated with 0.03% tacrolimus ointment for severe atopic blepharoconjunctivitis and had provided a conjunctival cytology sample prior to and within 3 months from the start of their treatment.

Concomitant treatments

Prohibited therapies in Studies I, II, and V included other investigational drugs, UV-light therapy, non-steroidal immunosuppressants, topical medicated agents, and the systemic antihistamines astemizole and terfenadine. In addition, no systemic and topical corticosteroids were allowed during the first 6 months of Studies II and V. After that, rescue therapy for AD exacerbations of a maximum of 2 weeks in every 3 months was allowed. Use of non-steroidal anti-inflammatory agents was restricted. Emollients were not allowed for 2 hours prior to or after application of tacrolimus ointment. Inhaled corticosteroids at doses up to 1 mg/day were permitted for treatment of bronchial asthma or allergic rhinitis; this was also valid for Study III. For wash-out periods see Table 4.

In Study IV, eye-drops containing sodium chromoglycate or its derivatives for conjunctivitis symptoms were allowed if use had preceded tacrolimus treatment. Prior to the first dose of tacrolimus ointment, all patients had been without topical or systemic antihistamines and glucocorticoids for at least 2 weeks.

Exclusion criteria

Exclusion criteria are presented in Table 5. In Study IV, exclusion criteria were concomitant topical or systemic antihistamine, glucocorticoid, or ciclosporin treatment or another significant disease affecting the ocular area.

Table 4. Wash-out periods for previous treatments before entering the studies.

Medication / Study	I	III	II, V
All medicated topical agents including corticosteroids	1 day	n.r.	5 days
Systemic antihistamines			
Terfenadine	n.r.	5 days	5 days
Astemizole	n.r.	6 weeks	6 weeks
Systemic corticosteroids	4 weeks	2 weeks	2 weeks
Systemic non-steroidal immunosuppressants such as ciclosporin, methotrexate	1 day	2 weeks	2 weeks
Other investigational drugs	4 weeks	4 weeks	4 weeks
Ultraviolet light treatments	1 day	n.r.	6 weeks

n.r. = not restricted

Table 5. Exclusion criteria for Studies I, II, III, and V.

Study	I	III	II, V
Any infection requiring treatment	x	x	x
Known hypersensitivity to macrolides or excipients of the study ointment	x		x
Skin disease other than AD requiring treatment	x	x	x
HIV positivity	x	x	x
Clinically significant impairment of renal or hepatic function	x	x	x
Use or likely need for prohibited medication during the study	x		x
Cancer or history of cancer or other systemic disease contraindicating use of tacrolimus	x		x
Patient is pregnant or breast-feeding	x	x	x
Clinically infected AD		x	x
History of eczema herpeticum			x
Other chronic uncontrolled or unstable disease		x	x
Substance abuse or a psychiatric disorder affecting co-operation		x	x
Simultaneous participation in any other drug trial			x
Acute or likely infection with chicken pox			x

Methods

Treatment with tacrolimus ointment

In Studies I, II, and V, patients used 0.1% tacrolimus ointment intermittently twice daily, on all AD affected areas. Patients were instructed to continue applying ointment for one week after cessation of itch and clearing of the AD lesions and to start the treatment similarly on areas where AD symptoms reappeared. In Study IV, tacrolimus 0.03% ointment was used on the affected eyelids once daily for the first 4 weeks and later intermittently depending on the individual response.

Assessment of atopic dermatitis

In Study I, the eczema score, erythema, induration, excoriation, lichenification, and scaling of the worst AD lesion were each graded on a scale of 0 to 3 and summed (maximum 15). Changes in eczema score were calculated from baseline to week one, month 6, and month 12 for each patient. In Studies II and V, both the efficacy and safety of treatment with tacrolimus were monitored, with patient visits at baseline (day one); weeks one and 4; and months 3, 6, 9, and 12. BSA was evaluated at baseline and at weeks one and 4 and months 3, 6, 9, 12, and in Study V every six months thereafter until month 48. The treatment response for AD was evaluated also by physician's and patient's global assessment (PGA), as in case report forms of the study, on how satisfactory the treatment had been for the patient's condition (excellent, very good, good, fair, or poor). In Study V, patients who sustained an excellent or very good PGA after month 3 were analyzed as a separate group of patients with the best treatment response. Adverse events and concomitant medications were followed. Laboratory assessments (hematology and clinical chemistry including assessments of renal and hepatic function) were performed on day one, at week one and months 6 and 12. In the conventional therapy group of Study II, the affected BSA was assessed at baseline and month 12. No laboratory tests were scheduled.

BSA was evaluated for all subjects once in Study III. In Study IV, a simple scale was created to measure the symptoms. The severity of blepharitis (scaling, lichenification, and excoriations of the eyelid skin, thickening of the lid margin, thinning of lashes) and conjunctivitis (hyperemia, swelling, upper tarsal follicles, and macropapills) were both assessed on a scale from 0 to 3 (no symptoms, mild, moderate, or severe). No keratitis score was included, as only three patients showed signs of mild epithelial punctate keratitis in the first assessment.

Staphylococcus aureus colonization

Assessments of staphylococcal colonization took place at baseline, weeks one and 2, and monthly thereafter. Agar plate lifts from the worst AD lesion of each patient for bacterial culture came from application of a contact plate (NUNC, Roskilde, Denmark) for 5 seconds. Cultures were incubated aerobically in a cystine-, lactose-, and electrolyte-deficient agar medium at 35°C for 24 hours. The following criteria were used to identify *S. aureus*: typical colony morphology on the contact plates and in pure cultures on a blood agar plate, a positive catalase test result, positive DNase test result, or a positive coagulase tube test result. The density of *S. aureus* colonies was expressed as colony-forming units per square centimeter (CFU/cm²). Changes in CFU/cm² were calculated from baseline to week one, month 6, and month 12 for each patient.

Skin collagen synthesis and skin thickness

Measurements of the tacrolimus patients and controls treated with conventional treatment were done at baseline and after 12 months of treatment. Healthy subjects underwent the measurement only at baseline. Measurements of the control groups were performed during the same time period as in the primary study. Collagen synthesis was assessed by measuring the level of aminoterminal propeptides of procollagen types I and III (PINP and PIIINP, respectively) from suction blister fluid samples of abdominal skin. The blisters were raised by a disposable suction blister device (Ventipress Oy, Lappeenranta, Finland) that draws interstitial fluid between the epidermis and dermis (Kiistala 1968). The fluid was aspirated from the blisters after approximately one hour of suction at -100 to -300 mmHg and frozen at -20 degrees of Celsius. Concentrations of PINP and PIIINP were determined by specific radioimmunoassays (Orion Diagnostica, Oulunsalo, Finland) (Risteli et al. 1988, Melkko et al. 1990, Oikarinen et al. 1992).

Skin thickness, defined as the distance between the surface of the stratum corneum and the lower dermis, was measured digitally with a high-frequency ultrasound device (DUB20-S, Taberna pro medicum, Lüneburg, Germany) (Tan et al. 1982). As skin thickness differs markedly for different areas of the body, the measurements were made in eight target regions: forehead, cheek, collarbone, thorax, upper arm, inside elbow, forearm, and the abdomen 2 cm left of the navel, independently of whether the skin was affected by AD or not. Measurements were taken at a frequency of 30 MHz, and results were calculated as the median thickness of the eight sites. Initially the plan was to measure skin thickness in all patients, but technical problems with the ultrasound device prevented a complete analysis, and participation or non-participation in skin thickness measurement was random.

Respiratory symptoms and findings

To exclude seasonal variation in respiratory symptoms, patients were enrolled from late October to early January after the pollen season and before the typical respiratory infection epidemics. Measurements were done once for the study groups in Study III, and at baseline and after 12 months of treatment in Study V.

Lung function tests

The forced expiratory volume in one second (FEV1) (Quanjer et al. 1993) was recorded with a flow-volume spirometer (Medikro 905, Medikro Oy, Kuopio, Finland), with results expressed as percentages of the national predicted values (Viljanen et al. 1982). Bronchial hyper-responsiveness was evaluated with a dosimetric histamine challenge test employing increasing inhaled doses of 0.025, 0.1, 0.4, and 1.6 mg buffered histamine diphosphate (Sovijärvi et al. 1993). Based on the dose-response curve, the provocative dose of inhaled histamine producing a decrease of 15% in FEV1 (PD15FEV1) was determined. According to PD15FEV1, bronchial hyperreactivity was graded as mild (0.41-1.60 mg), moderate (0.11-0.40 mg) or severe (<0.1 mg). Subjects with PD15FEV1 value >1.6 mg were considered to have normal bronchial responsiveness. Additionally, the result was expressed in terms of the slope of the dose response curve, as a continuous and log-normally distributed index (O'Connor et al. 1987). At the time of lung function testing, all patients were free from respiratory tract infections for at least the preceding 2 weeks, and short-acting beta-2-agonists were withheld for at least the previous 12 hours.

Questionnaire

A previously validated questionnaire was chosen for estimating the prevalence of physician-diagnosed asthma and allergic rhinitis (Haahtela et al. 1980). The degree of asthma symptoms (cough, wheezing, dyspnoea) and of nasal symptoms experienced by the AD patients during the preceding year were estimated on a visual analogue scale (VAS) from 0 to 10 cm. AD patients with symptoms of at least 1 cm in VAS in Study III and of at least 2 cm in VAS in Study V were considered to have current asthma or nasal symptoms. The patients in Study V answered the questionnaire also at 48 months of treatment. The control subjects in Study III gave qualitative answers as to any possible asthmatic or nasal symptoms.

Induced sputum

Sputum was induced by inhalation of 5 ml of 3% NaCl solution, with an ultrasonic nebulizer (Omron U1, Omron, Germany) for 15 minutes (Djukanovic et al. 2002, Metso et al. 2001). Subjects were pre-treated with 200 µg salbutamol (Buventol Easyhaler 100 microg/dose®, Orion Pharma), by inhalation. PEF values were measured before and

after induction, to ensure safety of the procedure. If PEF fell by more than 15%, or troublesome symptoms appeared, patients were treated with 200 µg of inhaled salbutamol. Subjects were asked to cough during and after inhalation, and sputum samples were collected in empty containers. The more viscous parts of the samples were collected and mixed for air-dried smears which were stained with eosin and methylene blue. Eosinophilia was graded semi-quantitatively, on a scale from 0 to 4 (Ryttilä et al. 2002), as relative proportions of eosinophils of all non-squamous cells: less than 1%, 1 to 5%, 6 to 10%, 11 to 50%, and >50%, respectively. In Study V, the cell proportion of grade 0 was determined as <2%. Grades 1 to 4 were considered to represent bronchial eosinophilia. A sample was considered to be adequate, and to originate from the lower airways, if it contained macrophages and less than 50% of squamous epithelial cells. All analyses were conducted blinded to the clinical characteristics of the subject.

Skin prick tests and serum IgE

Skin prick tests (SPT) including histamine dihydrochloride 10 mg/ml as the positive control, saline as the negative control, and ten allergens: birch, timothy, mugwort, cat, dog, cow, horse, *cladosporium herbarum*, *D. pteronyssinus*, and natural rubber latex (Soluprick SQ 10 HEP, ALK, Copenhagen) were performed once in Study III and at baseline and after 12 months in Study V on the skin of either forearm. A reaction of at least 3 mm in the presence of at least a 5-mm reaction to histamine dihydrochloride and a negative reaction to saline was interpreted as positive for the allergen in question. The patient was regarded as atopic if at least one allergen reacted positively (Dreborg 1989).

A blood test for serum total immunoglobulin E (s-IgE) was determined by a fluoroenzymeimmunoassay (ImmunoCAP™, Phadia AB, Uppsala, Sweden) once from all participants in Study III and at baseline and after 12 and 48 months of treatment in Study V. The levels observed were regarded as elevated if they exceeded the following age-related reference-range upper limits: over 17 years, 110 kU/L; 15-17 years, 160 kU/L; and 13-14 years, 320 kU/L (Björkstén & Viander 1987).

Conjunctival cytology

Conjunctival cytology samples had been collected with a special brush (Accellon® Multi Biosampler) from the lower lid conjunctiva of the both eyes (Kari 1988) before the first dose of tacrolimus and at the next follow-up visit. The cells were counted from smear preparations and staged on a scale from 0 to 4 (0=0; 1=1-10; 2=11-40; 3=41-60; 4=>60 cells per slide). The result was expressed as the mean count of both eyes.

Statistical methods

In Studies I, II, and IV, comparisons were carried out with the statistical software package SAS and in Studies III and V with the SAS Statview program. For continuous or normally distributed variables, such as log slope of the dose response curve, t-test or ANOVA was used. Normal distribution was assessed by histograms. For non-continuous variables or variables with non-normal data distribution, non-parametric tests were used (e.g. *S. aureus* colony forming units, BSA, IgE, asthma and rhinitis symptoms). The Mann-Whitney test served to test differences between groups and the Wilcoxon signed rank test for differences within a group over a period of time. The chi-square test (χ^2 -test), or Fischer's exact test served for nominal variables such as numbers of patients. Spearman's correlation analysis was used to estimate the relationship between the variables. Logistic regression served in Study III to analyze the relationship between signs of airway disease and AD. In Studies II and V, an intention-to-treat analysis with the last-observation- carried-forward method was performed as required by the distribution of the variable in question. A p-value less than 0.05 was considered statistically significant in all studies. Multiple comparisons in Study III had originally not been tested with a Bonferroni correction. Additional analyses with the Bonferroni correction applied later gave similar p-values and had no effect on the statistical significance of the results.

RESULTS

Clinical efficacy and safety

The clinical efficacy of topical tacrolimus treatment in the four studies was generally very good. The AD patients in Study III had a mean BSA of 29% (Table 6).

Treatments with tacrolimus 0.1% ointment in Studies I, II, and V, as well as with topical corticosteroids in the control group of Study II were well tolerated, and with no serious adverse events or significant laboratory abnormalities. The most common adverse event was a burning sensation in the tacrolimus-treated areas which usually disappeared during the first week of the treatment. Similarly, in Study IV, the most common adverse event was a transient burning or heat sensation in the treated area during the first treatment days. No ocular or eyelid area infections occurred during follow-up. No significant changes occurred in intraocular pressure, the cornea, lens, refraction, anterior chamber, or retina during the treatment with tacrolimus 0.03% ointment.

Table 6. Clinical response to tacrolimus ointment.

Study	Method	Subgroup	Baseline	Follow-up	Response (%)	p-value
I	Score median	6-month treatment	7.0	2.0	71	<0.001
		12-month treatment	7.0	0.0	100	0.008
II	BSA mean (median)	Patients	32.5 (21.3)	9.4 (3.5)	69.0 (90.0)	<0.0001
		>90% clearance (N)		27	48	
		Controls with AD	22.6 (17.5)	15.7 (7.8)	23.0 (55.0)	0.0019
		>90% clearance (N)		6	17	
IV	Score mean (median)	Blepharitis	3.0 (3.0)	1.0 (1.0)	67	0.003
		Conjunctivitis	2.3 (2.5)	0.6 (0.0)	74	0.008
V	BSA mean (median)	All*	30.6 (18.7)	10.6 (3.1)	65.8 (90.0)	<0.0001
		BTR** (N=36, 56%)	28.4 (17.0)	2.2 (0.2)	93.0 (99.2)	<0.0001
		48 months		9.5 (2.8)	68.1 (86.4)	0.0001

BSA=Body surface area of atopic dermatitis (%)

* Follow-up after 12 months

** BTR=Best treatment response in AD by physician's and patients' global assessments (PGA)

Staphylococcus aureus colonization

By the first week of the treatment, *S. aureus* colonization and the eczema score decreased markedly. The median decrease in CFU/cm² from baseline was 59.1 at week 1 ($p=0.012$), 102.4 at month 6 ($p<0.001$), and 234.9 at month 12 ($p=0.008$). Each decrease correlated with the improvement in the eczema lesions. The median decrease in the eczema score of the worst lesion from the baseline was 4.5 at week 1 ($p<0.001$), 5.5 at month 6 ($p<0.001$), and 8.0 at month 12 ($p=0.008$).

Skin collagen synthesis and skin thickness

At baseline, the median combined PINP and PIIINP levels in both the tacrolimus and conventional treatment groups were lower than in healthy controls, at 193.0 and 285.0 µg/L vs. 515.0 µg/L, respectively. During the course of the study, the synthesis of both collagen type I and type III increased with tacrolimus treatment. Levels of PIIINP rose by a median of 158.0 µg/L at 12 months, compared with baseline (+202.6%, $p<0.001$). PINP levels increased by 65.0 µg/L (+59.1%, $p<0.001$). The median combined levels of PINP and PIIINP increased by 272 µg/L (+140.9%, $p<0.001$). In contrast, patients receiving conventional therapy showed no significant change in median combined PINP and PIIIP levels over 12 months, with an increase in only 11.0 µg/L (+3.9%, non-significant). Differences in changes between the two treatment groups were significant for single and combined procollagen propeptide levels. PINP and PIIINP were measured again after 24 months for 46 patients who had continued using 0.1% tacrolimus ointment. Their values had decreased to a level similar to that of the healthy controls (Fig 1). An intention-to-treat analysis with all the original 56 patients gave similar results.

Median skin thickness at baseline for healthy controls was 1261.5 µm, for the tacrolimus group 1274.2 µm, and for the conventional therapy group 1355.4 µm. In the tacrolimus group, median skin thickness increased between baseline and month 12 by 114.7 µm (+9.0%, $p<0.001$). In contrast to these findings, skin thickness in the conventional therapy group by month 12 decreased by 110.7 µm (-8.2%, $p=0.001$). These differences in changes in skin thickness between the two treatment groups was significant ($p<0.001$). Three patients in the tacrolimus group showed clear visual signs of corticosteroid-related skin atrophy at baseline. In all of these patients, skin atrophy was progressively ameliorated during the tacrolimus treatment, as evaluated by collagen synthesis, skin thickness, and macroscopic signs of skin atrophy.

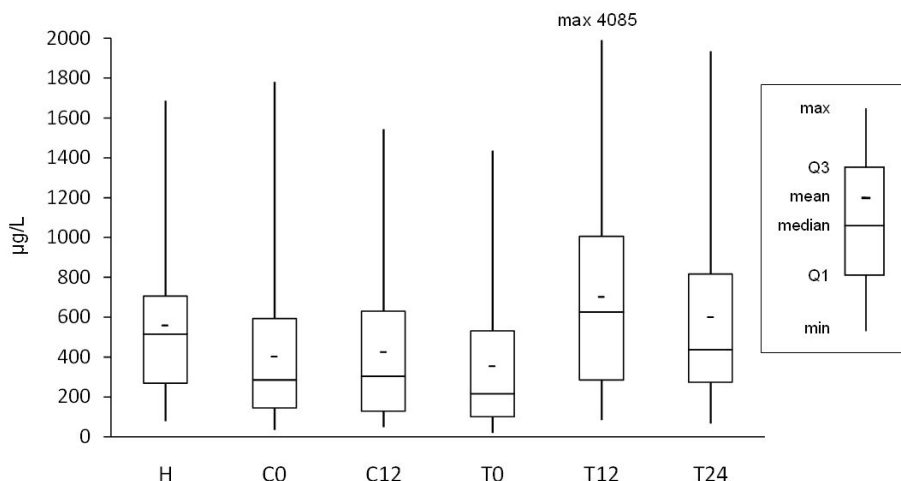


Figure 1. Combined levels of PINP and PIIINP in healthy controls and patients treated with corticosteroids or tacrolimus ointment 0.1% monotherapy. Mean \pm 95% CI, median, maximum, and minimum of H) healthy controls, N=27; C0) corticosteroid-treated patients, N=36, at baseline, and C12) after 12 months; T0) tacrolimus-treated patients, N=56, at baseline, T12) after 12 months, and T24) after 24 months.

Conjunctival cytology

In the conjunctival cytology samples, after treatment with tacrolimus ointment a significant decrease occurred in eosinophils (85%, $p=0.01$), neutrophils (58%, $p=0.02$), lymphocytes (50%, $p=0.01$), and squamous cell metaplasia (39%, $p=0.04$). No change occurred in epithelial, columnar, or goblet cells, nor was any cellular atypia evident. Of ten patients, eight used concomitant eye-drops containing sodium chromoglycate or its derivatives.

Respiratory symptoms and findings

In Studies III and V, most of the AD patients were SPT-positive. Life-time prevalences of physician-diagnosed asthma and allergic rhinitis were higher than in control subjects, but only a subgroup of patients was currently actively treated (Table 7).

Table 7. Subject characteristics; occurrence of atopy, physician-diagnosed asthma and allergic rhinitis in subjects of Studies III and V.

	Study III AD patients	Study III Control subjects	Study V AD patients
N	86	49	65
Females; N (%)	57 (66)	13 (27)	41 (63)
Smokers; N (%)	17 (20)	5 (10)	11 (17)
SPT-positive; N (%)	72 (84)	18 (37)*	53 (82)
Life-time prevalence of physician- diagnosed asthma; N (%)	31 (36)	1 (2)*	27 (42)
Current inhaled corticosteroids **	9 (29)	1	10 (37)
Current bronchodilators alone	18 (51)	0	15 (56)
Life-time prevalence of physician- diagnosed allergic rhinitis; N (%)	39 (45)	3 (6)*	29 (45)
Current intranasal steroids **	6 (15)	1 (33)	3 (10)
Current antihistamines	27 (69)	2 (67)	20 (69)

* p<0.01

** % of patients with diagnosis of asthma or allergic rhinitis

Respiratory symptoms

In Study III, patients with AD reported current airway symptoms, physician-diagnosed asthma and physician-diagnosed allergic rhinitis significantly more often than did the control subjects (Table 8). Current airway symptoms were significantly associated with BHR.

In Study V, asthma and rhinitis symptoms decreased significantly ($p=0.005$ and $p=0.02$) (Table 8). The change was more evident in patients with symptoms >2 cm in VAS scale at baseline (4.09 ± 1.6 vs. 2.43 ± 2.5 $p=0.003$ and 5.19 ± 2.39 vs. 3.36 ± 2.91 , $p=0.002$, respectively). At 48 months, a further but non-significant decrease compared with the 12-month results occurred to asthma and rhinitis symptoms (1.28 ± 2.23 and 1.93 ± 2.90). These changes were significant compared with baseline, $p=0.002$ and $p=0.005$.

Bronchial hyper-responsiveness

In Study III, increased BHR was observed significantly more often in AD patients than in control subjects, and degree of BHR, expressed as mild, moderate, or severe, or as the slope of the dose response curve, was more severe in the patients with AD than in controls ($p=0.006$) (Table 8). Of the 30 AD patients with previous or current physician-diagnosed asthma, 21 (70%) showed increased BHR. AD patients without asthma showed less BHR (40%) than did AD patients with asthma ($p=0.008$) but still more

compared with control subjects (10%, $p=0.001$). In terms of log slope of the dose response curve, BHR was significantly most severe in AD patients with asthma (3.17, range 0.92-6.6) than in AD patients without asthma (1.86, range 0.47-4.47, $p<0.0001$), or in control subjects (1.08, range -1.1 – 2.47, $p<0.0001$). Although the study groups differed slightly in terms of gender and smoking status, gender had no effect on occurrence or severity of BHR. Similarly, after excluding all the smoking subjects from both groups, the differences remained highly significant ($p<0.0001$).

In Study V, the number of patients with increased BHR decreased significantly ($p<0.0001$) (Table 8). A total of 13 (39%) of the 33 patients who showed BHR at baseline showed none at 12 months, and 3 (10%) of the patients with normal bronchial responsiveness at baseline showed mild BHR at 12 months. The log slope of the dose response curve of the histamine challenge test decreased significantly in patients with increased BHR at baseline (4.36 ± 1.19 vs. 2.88 ± 1.38 , $p=0.015$, t-test) as well as in patients with physician-diagnosed asthma (3.27 ± 1.50 vs. 2.68 ± 1.58 , $n=27$, $p=0.04$), i.e., representing a significant alleviation of BHR. The improvement in log slope of the dose response curve was most clear in patients with moderate-to-severe BHR at baseline (4.86 ± 0.90 vs. 3.58 ± 1.51 , $p=0.02$, $n=11$). Improvement in BHR was apparent in patients with no asthma medication or in those using bronchodilating agents only as needed but not in those using inhaled corticosteroids.

Induced sputum

In Study III, a representative sputum sample was available from 79% of the AD patients and 95% of the control subjects. Eosinophils were present significantly more often in the sputum of AD patients than in control subjects (Table 8). Grade of sputum eosinophilia was significantly higher in AD patients than in control subjects (1.30 vs. 0.11, $p<0.0001$). No significant difference appeared in the prevalence (83% vs. 80%) or grade (1.20 vs. 1.07) of sputum eosinophilia between AD patients with or without asthma.

In AD patients, smoking was significantly associated with sputum eosinophilia ($p=0.0012$). In a subanalysis of non-smoking subjects of both groups, this difference, however, remained significant for occurrence ($p=0.02$) and grade ($p<0.0001$) of sputum eosinophilia. In AD patients, sputum eosinophil counts were significantly associated with bronchial ($p=0.01$) and nasal ($p=0.02$) symptoms (VAS) but not with physician-diagnosed asthma or allergic rhinitis. Moderate or severe BHR was significantly more common in patients with sputum eosinophilia (grade ≥ 2) than in patients without sputum eosinophilia ($p=0.0007$).

In Study V, an acceptable sputum sample was obtainable from 49 (77%) patients. Sputum eosinophilia (grade 1-4) was detectable in 21 (43%) patients at baseline and in 20 (41%) after 12 months. Sputum eosinophils decreased in patients with no physician-diagnosed asthma (1.83 vs. 1.03, $n=18$, $p=0.005$) and increased in patients with

physician-diagnosed asthma (2.09 vs. 3.10, $n=11$, $p=0.03$). In patients with sputum eosinophilia at baseline and the best treatment results in AD, sputum eosinophils decreased significantly ($n=12$, $p=0.04$). No change in the count of blood eosinophils as such was evident (Table 8), but in the 18 patients with baseline blood eosinophilia ($>0.5 \times 10^9/L$), the number of blood eosinophils decreased (0.95 vs. 0.66, $p=0.003$).

Skin prick tests and serum IgE

In Study III, 72 (84%) AD patients and 16 (35%) control subjects showed at least one positive reaction in the SPT, ie. they were classified as having IgE-associated or allergic AD. The SPT- positive AD patients had significantly lower FEV1% than did SPT-negative patients (94.1 vs. 103.6, $p=0.02$). Physician-diagnosed asthma (31 vs.1, $p=0.01$), allergic rhinitis (37 vs. 3, $p=0.05$), and BHR (40 vs. 3, $p=0.02$), but not current airway symptoms or sputum eosinophilia, were significantly more common in SPT-positive AD patients. This was also true for AD patients with increased s-IgE ($n=59$, 69%) with the exception that increased s-IgE correlated with current nasal symptoms and sputum eosinophilia.

In Study V, no significant change emerged in serum IgE in the study population as such (Table 8). An almost significant decrease in serum IgE occurred in patients with elevated serum IgE at baseline and the best treatment results in AD (3690 ± 4390 vs. 2233 ± 2120 , $n= 25$, $p=0.06$, n.s.), whereas in patients with a less favorable AD response, serum IgE increased (3497 ± 5298 vs. 4226 ± 5004 , $n=22$, $p=0.10$, n.s). Serum IgE levels at 48 months were similar to the results at 12 months (4113 ± 14905 , n.s. compared with baseline).

A total of 53 (82%) patients were SPT-positive at baseline. At 12 months, SPT reactivity had increased significantly, expressed as number (3.9 vs. 4.1, $p=0.002$) and sum (21.2 vs. 25.1 $p<0.0001$) of positive reactions. The SPT reaction to histamine increased slightly (5.1 vs. 5.2, n.s.). One SPT-negative patient in the tacrolimus group had become SPT-positive at 12 months.

Table 8. Body surface area of atopic dermatitis (%), lung function, bronchial hyper-responsiveness, and sputum eosinophilia in subjects of Studies III and V.

	Study III AD patients	Study III Control subjects	Study V AD patients at baseline	Study V AD patients at 12 months
BSA (%); Mean \pm SD	29 \pm 24.4		30.6 \pm 25.3	10.6 \pm 17.1*
Serum IgE (kU/L)	2498 \pm 4157		2701 \pm 4377	2260 \pm 3469
Blood eosinophils (E9/L)			0.47 \pm 0.40	0.41 \pm 0.33
Current asthma symptoms; % (mean VAS/cm)**	53 (1.7 \pm 2.1)	16*	38 (1.9 \pm 2.2)	22* (1.4 \pm 2.1)*
Current nasal symptoms; % (mean VAS/cm)	67 (3.1 \pm 3.1)	16*	59 (2.8 \pm 3.0)	33* (2.0 \pm 2.7)*
FEV1%	95.7 \pm 11.6	94.4 \pm 12.3	96.1 \pm 10.3	94.9 \pm 9.9
Increased BHR; N (%)	43 (51)	5 (10)*	33 (52)	23 (36)*
Mild	28 (33)	5 (10)	22 (34)	15 (23)
Moderate or severe	15 (18)	0	11 (17)	8 (13)
Log slope	2.32 \pm 1.38	1.19 \pm 0.86*	2.37 \pm 1.52	2.10 \pm 1.43
Successful sputum samples	68	18	49	49
Sputum eosinophilia **	55 (81)	2 (11)*	21 (43)	20 (41)
Sputum eosinophils	1.2 \pm 0.9	0.1 \pm 0.3	1.2 \pm 1.0	1.20 \pm 1.3

BSA=Body surface area of atopic dermatitis (%)

VAS=Visual analogue scale

FEV1%=Forced expiratory volume in one second (% of predicted value)

BHR=Bronchial hyper-responsiveness

* p<0.05

** The criteria for symptomatic patients and sputum eosinophilia were different in Studies III and V.

Patients on inhaled corticosteroids

In the tacrolimus group of Study II, the nine patients who used inhaled corticosteroids for asthma had a mean AD affected BSA of 22.5% at baseline and 6.7% at 12 months. Four patients (44%) showed over 90% clearance at 12 months. The 47 patients using no inhaled corticosteroids had a mean AD affected BSA of 33.9% at baseline and 10.6% at 12 months. Signs of preclinical skin atrophy were more pronounced, and the median combined PINP and PIIINP level was lower at baseline in these patients than in the 47 patients using no inhaled corticosteroids: 129 μ g/L vs. 206.5 μ g/L. These baseline differences were substantially reduced following treatment with topical tacrolimus. At 12 months, median combined PINP and PIIINP levels were 695 μ g/L and 580 μ g/L, and for patients using and not using inhaled corticosteroids, the changes from baseline at 12 months were 490.0 μ g/L and 228.5 μ g/L.

In Study V, eight (12%) patients used regular and two intermittent inhaled corticosteroids at baseline. The dose of the medication remained unchanged during the study. Inhaled corticosteroid was started for one patient by a pulmonologist outside the study after 6 months of tacrolimus treatment, otherwise no changes occurred in asthma medication during the follow-up. The clinical response to tacrolimus was significant in this group as well: BSA decreased from 26.8 ± 16.7 to 8.4 ± 7.7 ($p=0.07$). Eight patients showed increased BHR at baseline. No significant changes in BHR, sputum eosinophils, respiratory symptoms, or serum IgE were detectable.

DISCUSSION

The main findings of four of the five studies (I, II, IV, and V) suggest that topical tacrolimus provides several favorable effects in the long-term treatment of AD. The clinical efficacy of topical tacrolimus was very good and sustained throughout the follow-up in all studies, with no marked safety issues, even after several years of intermittent monotherapy. Studies I, II, and V were conducted in the context of multicenter open-label long-term trials, the primary endpoint being the safety of topical tacrolimus in long-term treatment of AD. Although they included no randomized control groups, these studies are the first long-term studies to show significant improvement in staphylococcal colonization, suppression of skin collagen synthesis, and atopic respiratory symptoms, which are typical problems complicating AD and its treatment.

Staphylococcus aureus colonization

Superinfection with *S. aureus* is a common and potentially serious complication of AD, and *S. aureus* is believed to play an etiologic role in AD by release of superantigens. These superantigens are known to induce corticosteroid resistance by stimulating peripheral blood mononuclear cells. A different role for tacrolimus is supported by a study showing that FK506 (tacrolimus) inhibited SEB-induced peripheral blood mononuclear cell proliferation *in vitro*, whereas dexamethasone did not (Hauk & Leung 2001).

The present study discovered a significant and sustained decrease in *S. aureus* colonization of the AD skin. At present, only one 6-month study with mometasone furoate has shown a decrease in *S. aureus* colonization in cleared patients (Faergemann et al. 2000). However, in contrast to the present findings, the effect was most marked at 3 weeks of treatment, an effect that weakened thereafter. The present results are similar to those in two short-term studies, one concerning 0.1% tacrolimus ointment in adults (Pournaras et al. 2002) and one 0.03% tacrolimus ointment in children (Park et al. 2005). Tacrolimus has no inhibitory effect on bacteria, including *S. aureus*. The healing of skin lesions, as demonstrated by the improvement in eczema score and improvement in skin barrier function, probably contributed to the decrease in staphylococcal colonization. The reduction in *S. aureus* colonization over the long-term suggests that tacrolimus has local immunomodulatory activities in the skin. In agreement with this, clinical long-term studies with tacrolimus ointment to date have shown no evidence of increased risk for bacterial or viral skin infections (Hanifin et al. 2005, Remitz et al. 2007). Reduction in the staphylococcal trigger may benefit patients with AD in the long term.

Skin collagen synthesis

The results of Study II suggest that intermittent treatment with 0.1% tacrolimus ointment leads to a significant increase in skin thickness and in type I and type III collagen propeptide concentrations in the skin and a better clinical response than with corticosteroid treatment. The increase in collagen synthesis was reflected in a simultaneous increase in skin thickness. These results confirm the finding in a one-week study that topical tacrolimus does not suppress collagen synthesis (Reitamo et al. 1998).

Patients treated with 0.1% tacrolimus ointment showed a greater increase in PIIINP levels than in PINP levels, possibly indicating that different mechanisms regulate the synthesis of these two propeptides. Type III collagen is able to form intermolecular disulfide cross-links, which increase the mechanical stability of newly assembled collagen fibers, independently of the slower lysyl oxidase pathway usually needed in collagen assembly (Cheung et al. 1983). This ability benefits collagen-rich tissue requiring rapid growth and regeneration such as fetal skin, healing leg ulcers, and the repair site of ruptured Achilles tendons (Epstein 1974, Graham et al. 1984, Nimni 1983, Rasmussen et al. 1992, Eriksen et al. 2002, Liu et al. 1995). This phenomenon may also explain the increase of PIIINP in the skin no longer exposed to the suppressive effects of topical corticosteroids.

In the conventional treatment group, a one-year treatment period with corticosteroids had little effect on collagen synthesis, but the low procollagen propeptide levels at baseline were maintained. Simultaneous inhibition of collagen degradation may explain why collagen synthesis remains at a continuously low level once a certain degree of suppression is reached. This is supported by the finding that in addition to glucocorticosteroids, collagenase is inhibited also by inflammatory states such as atopic dermatitis by increase in tissue inhibitors of matrix metalloproteinases (Clark et al. 1987, Uitto et al. 1998, Katoh et al. 2002). Only a subgroup of patients of the present study had treated the actual site on the abdominal skin from which the collagen samples were obtained. Their procollagen levels in the SBF were similar to those of the rest of the patients, suggesting that the small, easily absorbed corticosteroid molecules actually have systemic effects in patients using an abundant amount of topical corticosteroids. In patients treated with tacrolimus ointment, procollagen levels increased from previously low levels of collagen synthesis to above the levels found in healthy controls, suggesting a rebound effect which then subsides after continued treatment of 24 months.

Inhaled corticosteroids reduce PINP and PIIINP levels in the skin of asthma patients (Autio et al. 1996). We also found this effect in our patients on inhaled corticosteroids. However, in our study, after one year of topical tacrolimus therapy the additional suppressive effects of inhaled corticosteroids on skin thickness and collagen synthesis were reversed.

The mode of action of tacrolimus is more specific than that of corticosteroids, as it targets NF-AT, a transcription factor expressed primarily in lymphoid tissues (Dumont 2000). No direct effect of tacrolimus on collagen-producing cells has been

reported, although one study showed tacrolimus to affect osteoblast differentiation and to increase collagen type III gene expression *in vitro* (Krocker et al. 2006). It therefore seems likely that a major factor for increased collagen synthesis in the tacrolimus group in our study was the discontinuation of topical corticosteroids, which have a wide range of target cells and tissues. Another factor contributing to increased collagen synthesis in patients using tacrolimus ointment may be an increased level of TGF- β , a cytokine enhancing collagen synthesis (Tsai et al. 2006). AD skin has been shown to present with subnormal levels of TGF- β (Arkwright et al. 2001, Caproni et al. 2006), tacrolimus to stimulate the expression of TGF- β in human T cells, peripheral blood mononuclear cells, and keratinocytes *in vitro* (Khanna et al. 1999, Lan 2004). In AD patients treated with topical tacrolimus for 3 weeks, TGF- β increased significantly in skin biopsies compared with levels in corticosteroid-treated skin (Caproni et al. 2006). This increase in TGF- β activity may lead to an increase in collagen synthesis by dermal fibroblasts. Furthermore, studies have suggested that FKBP-12, the receptor for tacrolimus, has inhibitory or modulatory effects on TGF- β family-mediated signalling by binding to the ligand-free TGF- β type I receptor. The TGF- β type I receptor is activated when FKBP-12 dissociates from it in the presence of high tacrolimus concentrations (Wang et al. 1996, Okadome et al. 1996).

Skin-thickness measurements have mainly been established for healthy skin (Reitamo et al. 1998, Haapasaari et al. 1995). In the present study, ultrasound measurements were performed on a subset of AD patients to evaluate this technique for diseased skin. At baseline, the tacrolimus group showed lower median skin thickness values than did the conventional therapy group and values similar to those of healthy controls. Treatment with tacrolimus ointment led to a significant increase in skin thickness, whereas conventional therapy led to a significant decrease to values slightly below those of healthy subjects. That inflammation and edema, features typically seen in patients with AD (Serup 1992), lead to increased skin thickness and mask corticosteroid-induced skin atrophy limits interpretation of these results. It can be assumed that the increased skin thickness seen at baseline was caused by inflammation, and treatment with corticosteroids may have led to atrophy and a reduction in inflammation, resulting in a decrease in skin thickness.

Treatment of eyelid dermatitis

Treatment of atopic blepharoconjunctivitis with 0.03% tacrolimus ointment was efficient without any marked adverse effects. In addition to marked improvement in the blepharitis, the conjunctival inflammation also resolved. In most patients, eyelid eczema improved significantly. Previous studies on tacrolimus ointment in atopic eyelid disease have shown a good response with no significant adverse events (Mayer et al. 2001, Rikkers et al. 2003), but the present study seems to have been the first to report its effects on conjunctival cytology.

Results of the present uncontrolled study on conjunctival cytology samples showed a clear decrease in the number of all inflammatory cells; this was in good agreement with clinical signs and subjective symptoms. Studies have shown that sodium chromoglycate or its derivatives inhibit mast cells and eosinophils to some degree in different types of allergic conjunctivitis. Nedocromil sodium reduced the concentrations of neutrophils, eosinophils, and lymphocytes in tear fluid by 72%, 88%, and 88% in patients with vernal conjunctivitis, but this finding did not reach statistical significance when compared with placebo (Bonini et al. 1992). After a conjunctival allergen challenge, lodoxamide-pretreated eyes showed 96% fewer eosinophils than placebo-treated eyes in tear fluid (Bonini et al. 1997). These substances are, however, insufficient to control the chronic atopic blepharoconjunctivitis in clinical practice. In our study their possible impact on clinical score or on cytological results was considered insignificant.

The modern antihistamine eye-drops have been shown to have anti-inflammatory properties in allergic reactions *in vitro* (Bielory et al. 2005). In placebo-controlled conjunctival allergen challenge settings *in vivo*, azelastine reduced counts of neutrophils, eosinophils, and lymphocytes in conjunctival scrapings in a conjunctival allergen challenge by 65%, 62%, and 82% (Ciprandi et al. 2003), and olopatadine-pretreated eyes showed 93%, 69%, and 80% fewer neutrophils, eosinophils, and lymphocytes in tear fluid than did placebo-treated eyes (Leonardi & Abelson 2003). Ketotifen has inhibited eosinophil function in an animal model of allergic conjunctivitis (Schoch 2003). At present, the effect of these agents on chronic atopic blepharoconjunctivitis remains unknown.

Tacrolimus eye-drops have inhibited the infiltration of eosinophils and lymphocytes as well as the late and delayed-type inflammatory response of experimental animal allergic conjunctivitis with an efficacy similar to that of betamethasone 0.1% and fluorometholone 0.1% eye-drops (Nishino et al. 2002, Sengoku et al. 2003). In the present study, tacrolimus was not applied directly to the eye. It is unclear how the treatment of the eyelids caused the improvement in the conjunctiva. The patients did not complain of any marked irritation as a sign of the ointment getting into the eyes. A systemic effect through such a small treatment area is unlikely. The most likely explanation is diffusion of the drug through the eyelid to the conjunctiva or that an improvement in the severe eyelid inflammation propagates to the adjacent conjunctival tissue.

Our clinical observations based on patients outside this study suggest a longer remission after the treatment period with tacrolimus ointment than with topical glucocorticoids, a good and well-maintained long-term treatment response, and also a diminished need for simultaneous eye- drop treatments. The decrease in the need to treat conjunctivitis separately, together with long-term disease control, is likely to improve patients' quality of life.

Atopic respiratory disease and topical tacrolimus treatment of atopic dermatitis

The results of Studies III and V suggest that respiratory symptoms, BHR, and eosinophilic airway inflammation are significantly more common in patients with AD than in control subjects without AD and that these decrease during effective long-term AD treatment.

BHR was associated with elevated s-IgE levels and skin prick test positivity, and sputum eosinophilia was associated with elevated s-IgE levels. BHR was present in 40% and airway eosinophilia in 18% of the patients without physician-diagnosed asthma. The prevalence of BHR in AD patients without asthma in previous studies has been 18 to 67%, depending on the method used and the age of the patients (Barker et al. 1991, Corbo et al. 1989, Price et al. 1976, Salob et al. 1993). Because airway inflammation and BHR may be signs of developing asthma, it is important to identify such patients for the early initiation of anti-inflammatory treatment. In addition, AD patients with physician-diagnosed asthma often seem to be undertreated, with no anti-inflammatory treatment. In our series, 50% of patients who were using only bronchodilating medication for asthma presented with moderate or severe BHR.

In Study V, intervention for AD with long-term intermittent topical tacrolimus resulted in a decrease in respiratory symptoms and BHR. This result is limited by our lack of a control group. However, to our knowledge, this is the first follow-up study on the effect of topical treatment of AD on respiratory symptoms. The study was not controlled, but can be regarded as a “real-life” study, which would be an appropriate way to study chronic diseases demanding long-term treatment (Bjermer 2006).

BHR measurements were not confounded by upper airway infections or the pollen season both of which may cause fluctuation in BHR over time. Furthermore, the study included subjects from a wide age-range, and the linear growth in adolescence might have explained the BHR improvement in only one patient. Reproducibility of the BHR measurement we used has been very good (Sovijärvi et al. 1993). Normalization of BHR occurred mostly in patients with originally mild BHR, but the largest decline in log slope of the dose response curve occurred in patients with moderate-to-severe BHR. Improvement in BHR was seen mainly in patients without ongoing inhaled or nasal corticosteroids, so the long-term effects of anti-asthmatic medication are an unlikely cause of the changes in BHR during follow-up. The simultaneous decrease in the respiratory and nasal symptoms supports the improvement in BHR and makes the results clinically more significant. Based on knowledge of the natural history of asthma and BHR, it seems that effective long-term treatment of skin inflammation in AD is associated with reduction in BHR and other respiratory signs beyond what can be expected from the natural variability of this disease.

S-IgE is a possible causative link between eczema severity, eosinophilic airway inflammation, and bronchial hyper-responsiveness. Based on the results of Study III, the patients with IgE-associated AD seem to have a clearly higher prevalence of atopic respiratory disease and its risk factors: BHR, positive skin prick tests, and sputum eosinophilia. Increased BHR has been reported in subjects with high s-IgE (Cookson et

al. 1986, Burrows et al. 1989, Kono et al. 2001) or positive SPT (Witt et al. 1986). AD severity is known to correlate with s-IgE (Wütrich 1978, Laske & Niggemann 2004), and IgE-associated AD is a risk factor for allergic asthma (Sears et al. 1991). In the present studies, BHR and airway inflammation were found to a lesser extent also in patients with normal s-IgE levels or negative skin prick test reactions. However, previous studies have indicated that airway eosinophilia and BHR are not completely dependent on s-IgE but rather on T cell pathways (Mehlhof et al. 1997, Hogan et al. 1997, Gavett et al. 1994, Riffo-Vasquez et al. 2000). In Study V, improvement in AD and respiratory parameters occurred to a similar extent in both IgE-associated and non-IgE-associated AD, but the group of patients with non-IgE-associated AD was too small (only ten) to make definite conclusions possible as to differences between groups.

Several studies suggest that epicutaneous antigen exposure can promote systemic allergic responses and lead to airway hyper-responsiveness. These effects can be seen especially in barrier-disrupted skin. Tacrolimus ointment suppresses T cell-induced inflammation efficiently and has favorable effects on the barrier function of the skin by normalizing TEWL and reducing staphylococcal colonization. Tacrolimus has shown suppressive effects on the keratinocytes (Lan et al. 2004), which in part initiate the inflammation in barrier-disrupted skin (Leung et al. 2004). In addition, tacrolimus ointment reduces the expression of several chemokines such as CCR3 (Park et al. 2005), which has been shown to play an important role in eosinophil recruitment to the skin and lung as well as in development of airway hyper-responsiveness (Ma et al. 2002). The systemic effect of tacrolimus is unlikely to explain the present results because systemic exposure to topical tacrolimus decreases along with skin improvement. The highest blood concentrations after topical tacrolimus treatment have been approximately 3% of those of transplant patients on systemic tacrolimus (Rubins et al. 2005).

The most likely explanation for our results is resolution of the cutaneous T cell inflammation and inhibition of the systemic T cell responses which originate from the skin and are capable of affecting the airways. Additionally, improvement in the skin barrier could reduce the penetration of antigens through the skin and thus inhibit systemic sensitization. Further support for the idea that topical tacrolimus can improve not only the skin barrier function in AD but consequently also other functional impairments of the skin is our finding of an improvement in the weakened or anergic recall antigen reactions of AD patients (Mandelin et al., in press), suggesting normalization of the T cell profile (Caproni et al. 2007). This would also explain the decrease in s-IgE levels in those patients who showed the greatest improvement in AD. The increase in number and sum of positive SPT results during this study may also indicate normalization of skin reactivity, although the repeatability of the SPT is not very good, and duplicate tests would have produced more reliable results (Dreborg 1989). However, in this study the effect was indeed systematic.

In summary, the present studies suggest that replacing topical corticosteroid treatment with long-term intermittent topical tacrolimus treatment for AD is not associated with the adverse events common during corticosteroid treatment, ones such

as skin atrophy. The results also suggest normalization of the structure and the function of the skin during this treatment. These effects are not restricted to the skin, because improvement in respiratory atopy may also be achieved. These results have special importance to patients with moderate-to-severe AD—the patient group most likely to suffer from the adverse events of topical corticosteroids, undertreatment of AD, or respiratory atopy.

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ERRATA

Page 36: In Table 2 in the column “Method”:

Third cell (Haapasaari et al. 1998) should be: SBF
 Serum

Fourth cell (Koivukangas et al. 1995) should be: SBF

Page 41: The following paragraph is missing:

Control groups

In study II, two control groups were matched demographically and clinically to the primary study group. Outpatients from our clinic with moderate-to-severe atopic dermatitis who were undergoing long-term intermittent treatment with topical corticosteroids, mostly of intermediate potency, comprised the conventional therapy group. In this group, a treatment period of two to three weeks was followed by at least a one-week period without treatment when clinically possible. Some of these patients received additional treatments such as ciclosporin (3 patients) and UV-light therapy (2 patients). Twenty-seven healthy subjects with no known skin or other diseases were used as controls representing normal skin.

In study III, a randomly selected group of 49 volunteer medical students or personnel of similar age but without AD or current airway symptoms were used as control subjects. Data on age, sex, smoking status, atopy status by skin prick tests (SPT), and medical history were collected from all subjects. Lung function tests were performed to all 49 and induced sputum to a subgroup of 19 volunteer control subjects.

Page 49: The correct legend for Figure 1 is:

Figure 1. Combined levels of PINP and PIIINP in healthy controls and patients treated with corticosteroids or tacrolimus ointment 0.1% monotherapy. H) healthy controls, N=27; C0) corticosteroid treated patients, N=36, at baseline and C12) after 12 months; T0) tacrolimus treated patients, N=56, at baseline, T12) after 12 months, and T24) after 24 months.